Arpp19 and Cdc6, two major regulators of the meiotic division in the Xenopus oocyte

Enrico Maria Daldello

Université Pierre et Marie Curie

Complexité du vivant

Institut de Biologie Paris Seine (IBPS)

Laboratoire : Biologie du développement

Equipe : Biologie de l'Ovocyte

Arpp19 et Cdc6, deux régulateurs majeurs des divisions méiotiques de l'ovocyte de Xénope

Par Enrico Maria DALDELLO

Thèse de doctorat de Biologie du Développement

Dirigée par Olivier HACCARD

Co-dirigée par Aude DUPRE

Présentée et soutenue publiquement le 12 juin 2015

Devant un jury composé de :

LORCA Thierry, Directeur de Recherches CNRS	Rapporteur
MECHALI Marcel, Directeur de Recherches CNRS	Rapporteur
DUPRE Aude, Chargée de Recherches CNRS	Examinateur
MULNER Odile, Directrice de Recherches CNRS	Examinateur
PINTARD Lionel, Directeur de Recherches CNRS	Examinateur

Université Pierre et Marie Curie

Complexité du vivant

Institut de Biologie Paris Seine (IBPS)

Laboratoire : Biologie du développement

Equipe : Biologie de l'Ovocyte

Arpp19 et Cdc6, deux régulateurs majeurs des divisions méiotiques de l'ovocyte de Xénope

Par Enrico Maria DALDELLO

Thèse de doctorat de Biologie du Développement

Dirigée par Olivier HACCARD

Co-dirigée par Aude DUPRE

Présentée et soutenue publiquement le 12 juin 2015

Devant un jury composé de :

LORCA Thierry, Directeur de Recherches CNRS	Rapporteur
MECHALI Marcel, Directeur de Recherches CNRS	Rapporteur
DUPRE Aude, Chargée de Recherches CNRS	Examinateur
MULNER Odile, Directrice de Recherches CNRS	Examinateur
PINTARD Lionel, Directeur de Recherches CNRS	Examinateur

Acknowledgments

First of all, I want to thanks Thierry Lorca and Marcel Mechali for having generously accepted to review my manuscript and be part of my jury in Paris. I'm also deeply grateful to Odile Mulner and Lionel Pintard for having agreed to participate to my Ph.D. committee as examiners.

I'm indebted to all the people in the lab for having welcomed me. I now realize that the time has passed so quickly that it is still difficult for me to believe that four years have gone by. A special thanks goes to the two directors of our laboratory, Catherine and Olivier, who have allowed me to pursue my Ph.D. in their lab. I thank Olivier for the many profitable discussions we had: the contribution of his experience and his tools has been fundamental. I also want to thank Catherine for her patient, inspiring, and insightful supervision of our project, and as well as for having sacrificed precious hours of sleep to correct my report, even though she was extremely busy. In addition, I am grateful to René to have been always available to discuss with me the results.

Naturally, the biggest thanks goes to Aude, who has supervised my daily work, and has always made herself available to help and support me even when nothing seemed to be working. She was the best supervisor I could hope for (and surely a better "supervisor" than a "ping pong player"!).
Her assistance has proved essential during these 4 years, especially in the complicated period where the Ph.D. manuscript was being prepared. I'm sure this "race against time" changed both of us permanently.
She became allergic to the sentence: "Do you have 5 minutes to discuss with me?".
I'm really sorry for that! You will never hear it anymore (at least not from me). However, a lot of synonyms exist: talk about, debate, exchange observations, etc. And now that I speak a bit of French, I can even change the language to ask the same.
On my side, I have become intolerant to the "red pen" she was using to correct my work. Fortunately, when told her about my distress, she immediately changed colour for the following hundred versions of the manuscript. Afterwards, my draft looked like a rainbow. To be honest, that was actually a nice way to add some colour to the black&white daily life of a biochemistry lab.
(ATTENTION, put away your RED PEN! The layout of this paragraph is not a mistake, it has been done as it is for a reason: this way, you have no space for corrections!)

Another special thanks go to Robert (*detto anche Roberto Pollo*). First of all, I really appreciate and have not forgotten that, since day one, he started to speak to me in English, an extremely costly effort for a native speaker of French! He has been the perfect "office mate": always ready to engage in serious discussions, but also able to joke, make days lighter and funnier (truth be told: we share a much similar humour). He has patently shared his immense experience as a biochemist, teaching me how to make perfect polyacrylamide gels (Aye, master! I have to admit that your gels are still better than mine). Finally, I am grateful to him for making me discover the "Ile Saint-Louis", and the so-called best "Tarte au citron" of Paris, which fed me for many days.

Un autre grand merci doit aller au Tran: tout d'abord d'avoir catalysé mon passage à la langue française. En six mois avec elle, je ai appris à parler beaucoup plus que ce que je pouvais dans les trois années précédentes. En son honneur je décide d'écrire ses remerciements en français. Ce fut un grand plaisir pour moi de travailler tous les jours avec elle à la paillasse. Nous avons formé une grande équipe, elle a apporté sa précision, moi la capacité de faire des calculs sans la calculatrice. Je la remercie également pour les belles discussions qui nous ont permis de survivre aux manips sans fin que nous avons faites pour le « projet Cdc6 » et d'avoir joué au ping-pong avec moi dans la soirée alors que nous attendions la maturation des ovocytes, qui étaient vraiment lente. Enfin je veux présenter mes excuses pour tout les moments où je lui ai volé ou matérielle de paillasse: mais je suis sûr qu'elle comprenait ma politique « Comunist » sur ce point particulier.

Thanks to Elisabeth for her help. I would like to mention the funny week in which, together with Tran, we tried to prepared the "kiwi" solution, which will be remembered as the most difficult solution ever crafted by mankind. In addition, I thanks the new entry of the lab Marika, for the kind suggestion on the preparation of the thesis oral presentation. Finally, thanks to "the two-halves", Melanie and Sandrine, for the nice chats and useful suggestions on how to pronounce French words in order to avoid embarrassing situations (e.g., the word "achat"), , and for having been among the most faithful fans of all the cakes, biscuits, drinks, and other culinary experimentations I have brought to the lab.

LIST OF ABBREVIATIONS

AC	Adenylate Cyclase
APC	Anapahse Promoting Complex
Arpp19	cAMP-phosphoregulated protein19
ARS	Autonomously replicating sequence
ATM	Ataxia Telengiectasia
ATR	ATM related
Bub	Budding uninhibited by benzamidazole
C-TAK	Cdc25C-associated protein kinase
CAK	CDK activating kinase
CaM Kinase II	Ca2+/calmodulin-dependent protein kinase II
cAMP	Cyclic Cyclic adenosine monophosphate
Cdc	Cell division cycle
Cdh1	Cdc20 homolog 1
CDK	Cyclin dependent Kinase
Cdt1	Cdc10-dependent transcript 1
Chk	Checkpoint kinase
CHX	Cycloheximide
Cip1	CDK inhibitor protein 1
CKI	CDK inhibitors
CPE	Cytoplasmic plyadenylation element
CPEB	CPE binding protein
CRL4	cullin ring E3 ligases 4
CSF	Cytostatic factor
CT	Cytoplasm Transfer
D-box	Destruction box
DDK	Dbf4-dependent kinase
DSB	Double-strand beark DNA
Emi2	Early mitotic inhibitors
ENSA	α-Endosulfine
ERK	Extracellular regulated kinase
Erp1	Emi1-releated protein 1
FEN1	Flap endonuclease 1
GST	Gultathion S trasferase
GVBD	Germinal Vesicle Breakdown
Gwl	Greatwall
IBMX	3-isobutyl-1-methylxanthine
IC	Initiation complex
Mad	Mitotic arrest defect
MAPK	Mitogen Activated Protein Kinase
Mastl	Microtubule associated serine/threonine kinase-like
MCC	Mitotic checkpoint complex
MCM	Mini Chromsome maintenance proteins
MEK	MAPK Erk kinase
MI	Meiosis I

MII	Meiosis II
MPF	M-phase promoting Factor
MpP	Maturation phosphorylated protein
Myt1	Membrane-associated tyrosine-and threonine 1
NEBD	Nuclear Envelop BreakDown
Nek	NIMA-related espressed kinase
NES	Nuclear localisation signal
NLS	Nuclear localization signal
OA	Okadaic acid
ORC	Origin Recognition Complex
p90 Rsk	p90 ribosomal S6 kinase
PDE	Phosphodiesterase
Pg	Progesterone
PIP-Box	PCNA-interacting protein box
PKA	cAMP-dependent protein kinase
PKA-C	Catalytic subunit of cAMP-dependent protein kinase
PKA-R	Regulative subunit of cAMP-dependent protein kinase
PKI	PKA inhibitor
Plx1	Polo like kinase 1
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
pRB	Retinoblastoma protein
Pre-RC	Pre Replicative Complex
Ringo	Rapid inducer of G2/M in oocytes
SAC	Spindle Assembly Checkpoint
SCF	Skp, Cullin, F-box containing complex
Sgo	Shugoshin
ssDNA	SIngle-stranded DNA
UTR	UnTranslated Region
xPR	Xenopus Progesterone Receptor

Table of contents

iii. ***Recombination and post-recombination events***

iv. ***The succession of two M-phases with no intervening S-phase***

v. ***The separation of homologous chromosomes***

b. Oogenesis and meiotic maturation

i. ***The oocyte growth period***

ii. ***Meiotic maturation***

1. **Morphological events**
2. **Profile of Cdk1 activity**

c. The arrest in prophase

i. ***cAMP and PKA activity***

ii. ***Status of Cdk1 and its direct regulators***

1. **Wee1 and Myt1**
2. **Cdc25**

d. Mechanisms controlling the release from the prophase arrest

i. ***The competence to resume meiosis***

ii. ***Hormones and Receptors***

iii. ***The limiting steps for meiosis resumption***

1. **The decrease of cAMP levels and the downregulation of PKA**
2. **The protein synthesis**
   - ***The Cyclins***
     - Cyclin A
     - Cyclins B
   - ***The kinase Mos***
   - ***Ringo/Speedy***
3. **PP1**

iv. ***The two-steps mechanism of Cdk1 activation***

1. **Formation of the starter amount of active Cdk1**
   - ***From monomeric Cdk1: the synthesis of Cyclins B***
   - ***From the pre-MPF: the synthesis of Mos, Myt1 and Cdc25***
2. **The MPF autoamplification loop**
   - ***Role of Cdk1 on its regulators: the core part***
   - ***Cdc25, Plx1 and PP1***
   - ***The Mos/MAPK pathway***
     - Effects on Cdc25
     - Effect on Myt1
   - ***Greatwall and PP2A-55δ***

e. The MI-MII transition

i. ***The role of Cdk1***

ii. ***Function of the Mos/MAPK pathway***

b. **Cdc6 is stable in prophase and is degraded in a SCF^{Cdc4}-dependent-manner in response to progesterone**
c. **Ectopic Cdc6 is turning-over during the MI-MII transition**
d. **The accumulation of endogenous Cdc6 is under the dual control of Cdk1-Cyclin B and the Mos/MAPK pathway during the MI-MII transition**
e. **Overexpressing Cdc6 blocks Cdk1 reactivation and drives the oocyte into a replicative interphasic state after meiosis I**
f. **A new model for the regulation of Cdc6 during the meiotic divisions**

F. Discussion

a. **A tiny window between the initial activation of Cdk1 and GVBD to get rid out Cdc6**
b. **The Mos/MAPK pathway: a Godfather for Cdk1 between the two meiotic divisions**
c. **What roles for the interaction between Cdc6 and Cyclin B ?**
    i. **The phosphorylation status of Cdc6**
    ii. **The specific binding of Cdc6 to different Cyclins B isoforms**
    iii. **Different status of Cdk1-Cyclin B phosphorylation**
    iv. **Localization of Cdc6 and Cdk1-Cyclin B complexes**
d. **High molecular weight complexes contains both MPF and Cdc6**

The overall goal of my project was to unravel the mechanisms responsible for the differentiation of a female diploid germ cell into a haploid fertilizable oocyte. Two critical features underlie the differentiation of female germ cells, turning it into a fertilizable oocyte able to support sexual reproduction. Firstly, the oocyte halts its meiotic progression in prophase of the 1st meiotic division, an arrest common to all species in the animal kingdom and necessary to support an intensive cell growth due to the accumulation of nutrients and determinants required for the early development of the future embryo. Secondly, the ploidy of the cell has to be reduced by half in order to generate a haploid gamete. This process is ensured by the succession of the two meiotic divisions with no intervening S-phase and no DNA replication in-between.

During my PhD, my scientific researches have been focused on these two specific subjects by using a powerful model system well suited for the analysis of meiotic divisions, the *Xenopus* oocyte. Meiotic divisions have evolved from the mitotic cell cycle, re-using molecular engines of cell proliferation, but placing them in a new context of regulatory pathways. Indeed, many actors of the meiotic divisions are conserved and shared with the mitotic cell cycle. That is why I chose to describe in the introductive part of this manuscript the control of the mitotic cell cycle. Then I highlight more specifically the mechanisms that control the meiotic divisions. This introductive part is followed by two chapters presenting my results. The first one is devoted to the small protein Arpp19 and its involvement in the prophase arrest. The second one focuses on the regulation of the Cdc6 protein, an essential replicative factor, and its role in controlling Cdk1 activity as well as DNA replication during the meiotic divisions. The manuscript ends with general perspectives of research avenues open by my results.

CHAPTER I:
Mitosis versus meiosis

I - Mitosis versus meiosis

In all living organisms, the universal way to propagate life is cell division that requires the faithful duplication of the DNA (DNA replication) followed by the equal segregation of the genetic information in two separate daughter cells (Cell division). In prokaryotes, which lack nucleus, mitochondria and other membrane-surrounded organelles, the cell division consists in a quick phase of DNA synthesis followed by the fission of the cell in two daughter cells. Eukaryotic cells possess a more complex structure than prokaryotes: the cytoplasm is partitioned in several specialized organelles and the genetic information is encoded by more than one chromosome encapsulated in the nucleus. Because these structures need to be shared between the two daughter cells, the cellular division called Mitosis is part of a highly regulated system, the mitotic cell cycle. This cycle orchestrates in time and space the replication of the DNA with the physical division of the cell and supports the asexual reproduction of single-cell organisms as well as the proliferation of somatic cells in multicellular organisms. Eukaryotes have also evolved a second type of cell division called Meiosis. In multicellular organisms, the meiotic division is specific of germ cells (sperms and oocytes) and consists in the succession of two cellular divisions, the meiosis I and the meiosis II, with no intervening S-phase or DNA replication. This specialized division allows the formation of haploid gametes that support the sexual reproduction. Following fertilization, the fusion of the oocyte with the sperm reconstitutes a diploid genome to originate the next generation.

A. The mitotic cell cycle

a. Overview of the cell cycle

The mitotic cell cycle is divided in two main phases: the interphase, during which the DNA is replicated and the M-phase, during which the cell physically divides. Beside S-phase, the interphase also contains two Gap periods, G_1 and G_2, occurring before and after the S-phase (**Fig. 1**). The G_1 phase marks the irreversible commitment of the cell within the cell cycle, which is regulated by extracellular mitogenic factors and the cellular size. During G_2, the cell synthesizes and assembles all the components and structures that will be necessary for the progression through M-phase. After fertilization, the embryonic cell cycles are characterized by a quick succession of S-phases with mitosis in the absence of G_1 and G_2 phases. This allows a very fast cell proliferation. G_1 and G_2 phases appear at time of the mid-blastula transition (MBT), also corresponding to the first transcriptional expression of the embryonic genome. Hence cell cycles start to be connected with external stimuli. Mitosis is the shortest phase of the cell cycle and is divided into five well-characterized periods: prophase, prometaphase, metaphase, anaphase and telophase. In prophase, chromosomes start to condensate inside the nucleus and the centrosomes separate from each other, prefiguring the poles

of the future mitotic spindle. In prometaphase, kinetochores are assembled on centromeres and get attached to the spindle microtubules in order to pull the chromosomes toward the middle of the cell. At this stage, the nuclear envelope breaks down. During metaphase, the chromosomes are aligned halfway between the spindle poles on the metaphase plate. In anaphase, the sister chromatids are split apart and migrate toward the opposite poles of the cell due to the shortening of the kinetochore microtubules and to the elongation of the spindle, that increases the separation of the chromosomes between the poles. During telophase, chromosomes reach the poles, the nucleus starts to reassemble around each group of chromosomes and the DNA decondensates. The telophase ends with the physical division of the two new-born cells during the process of cytokinesis.
In adult organisms, most of the cells are not engaged within the cell cycle but are arrested in a particular resting state called G0 (**Fig. 1**). The G0 arrest can be temporary (quiescent cells) as for stem cells, that are ready to re-enter into the cell cycle in response to external stimuli, or irreversible such as following the cellular differentiation or the entry into senescence.

b. Cell cycle engines

i. CDKs-Cyclins

The core components of the molecular cell cycle engine are Cyclin-dependent kinases (CDKs), which belong to the CMGC (Cdk1, MAPK, Gsk, CK) group of kinases. This family comprises 20 members from Cdk1 to Cdk20 that are involved in regulating transcription and cell cycle progression. CDKs are little kinases of about 250 amino acids residues (around 34-40 kDa) possessing a two-lobed structure. The amino terminal lobe, which is mainly composed of β-sheets, contains a Glycine-rich inhibitory element (G-loop) and a major helix (C helix) containing the PSTAIRE sequence of Cdk1. The carboxy terminal lobe, which is rich in α-helices, contains a flexible sequence that binds ATP (P-loop), the catalytic cleft and the activatory segment (T-loop). As monomers, CDKs are inactive because the catalytic cleft is closed by the T-loop, preventing the access to the ATP and thus their enzymatic activity.
The activation of CDKs requires their binding to activatory proteins, Cyclins (Jeffrey *et al.* 1995). Cyclins are a large family of approximately 30 members whose molecular weight varies from 30 to 90 kDa (Malumbres 2014). These proteins are structurally defined by the presence of a Cyclin-box, a large domain of 100 amino acids forming five α-helixes and mediating their interaction with CDKs (Kobayashi *et al.* 1992). Upon phosphorylation at T161 in the T-loop and the association of Cyclins to the C-helix of CDKs, CDKs sustain a conformational change that takes the PSTAIRE sequence out from the catalytic cleft. This process stabilizes the Cyclin-CDK heterodimer and opens the catalytic cleft to allow the binding of ATP within the P-loop (Jeffrey *et al.* 1995, Pavletich 1999, Echalier *et al.* 2014).

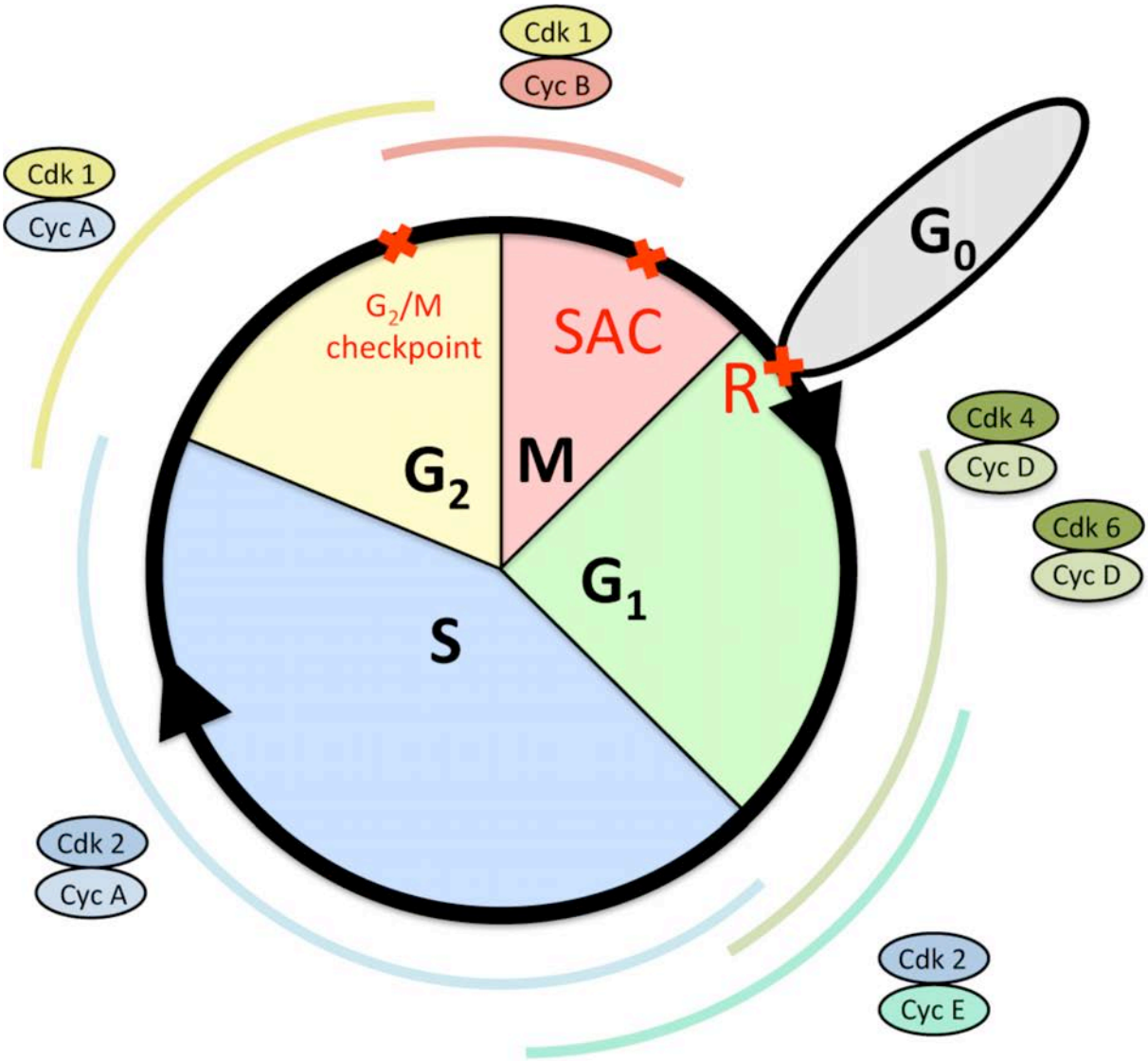

Fig. 1: The Cell Cycle

The cell cycle is controlled by the activation of different CDKs through the expression of different types of Cyclins. G_1-phase Cyclin-CDK complexes (Cdk4/6-Cyclin D/E) are important for the progression in G_1 and the commitment in S-phase. S-phase Cyclin-CDK complexes (Cdk2-Cyclin E and Cdk2-Cyclin A) are responsible for initiating and completing DNA replication. M-phase Cyclin-CDK complexes (Cdk1-Cyclin A and Cdk1-Cyclin B) drive entry and progression into M-phase. The progression within the cell cycle is regulated by checkpoint mechanisms: the Restriction point (R)at the begin of G_1, the G_2/M checkpoint and the SAC (Spindle assembly Checkpoint) in metaphase.

While there is only one CDK, the homolog of Cdk1 in yeasts (CDC28 in *S. cerevisiae* and Cdc2 in *S. pombe*) (Nurse *et al.* 1981, Stern *et al.* 1996), several CDKs are involved in cell cycle control metazoans (in mammals: Cdk1, Cdk2, Cdk4 and Cdk6 functioning as cell cycle engines) (**Fig. 1**). The same is true for Cyclins: in yeasts, two types of Cyclins, Clb (B type) and Cln, are present while metazoans have A, B, C D and E type Cyclins. Various combinations between CDKs and Cyclins generate complexes with different substrate specificities and therefore result in different cell cycle events (**Fig. 1**). G_1-phase Cyclin-CDK complexes such as Cdk4/6-Cyclin D are important for the progression through G_1 and the commitment in S-phase. S-phase Cyclin-CDK complexes such as Cdk2-Cyclin E and Cdk2-Cyclin A are responsible for initiating and completing DNA replication. M-phase Cyclin-CDK complexes such as Cdk1-Cyclin A and Cdk1-Cyclin B drive entry and progression into mitosis (**Fig. 1**).

ii. Regulation of CDKs-Cyclins

The control of CDKs genes expression plays a minor role in the regulation of CDK activity. For example, Cdk1 and Cdk2 are expressed at constant level all over the mitotic cell cycle. However, their activity is tightly regulated by at least three mechanisms: Cyclins availability, stoichiometric inhibition by CDK Inhibitors (CKIs) and phosphorylations (**Fig. 2**).

1. Association to Cyclins

The association of Cyclins with CDKs is mandatory for CDK activity. Since Cyclin levels are tightly regulated by transcriptional/translational mechanisms and by degradation, Cyclin levels constitute an important regulatory element of CDK activities. Cyclins are targeted to the proteasome by two ubiquitin ligases: SCF (Skp1-Cullin-F box protein complex) and APC (Anaphase Promoting Complex) (**Fig. 2**). The SCF recognizes Cyclins as substrates when they are phosphorylated at specific sites, whereas Cyclin ubiquitination by the APC occurs following the binding of two specific APC co-activators: Cdc20, at the anaphase onset and Cdh1 in late mitosis/G_1 transition.

2. Stoichiometric inhibition by CKIs

CKIs are subdivided into two classes based on their structure and CDK specificity. CKIs inactivate CDK catalytic activity by forming trimers with CDK-Cyclin complexes (**Fig. 2**). Yeasts possess a single specific inhibitor, Sic/Rum1, whereas higher eukaryotes utilize many inhibitors from the INK family

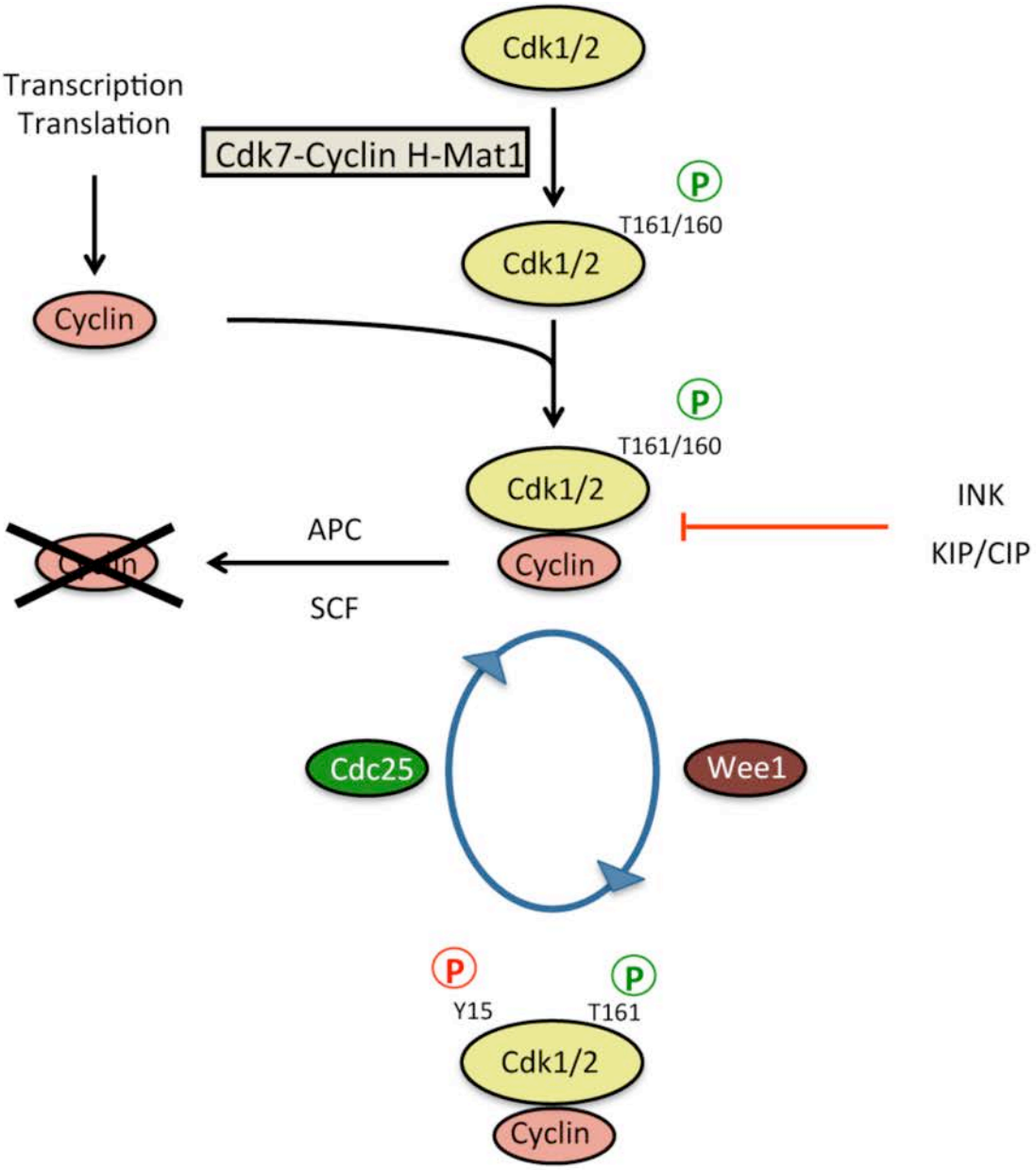

Fig. 2: Overview of CDKs regulation during mitosis

CDKs activity can be regulated mainly by three mechanisms: Cyclin availability, stoichiometric inhibition by CDK Inhibitors (CKIs) and positive/negative phosphorylation. Cyclin availability is tightly regulated by their degradation under the control of two E_3-ubiquitin ligases: SCF and APC (Anaphase Promoting Complex). CKIs from the INK and Kip/Cip families inhibit CDK-Cyclin complexes by direct binding. CDK-Cyclin complexes are further regulated by phosphorylation: an activatory phosphorylation at T161 (Cdk1) and at T160 (Cdk2) under the control of Cdk7-Cyclin H-Mat1 and an inhibitory phosphorylation at Y15. The phosphorylation at Y15 depends on the equilibrium betweenthe activities of the kinase Wee1 and the dual phosphatase Cdc25.

(Inhibitor of CDKs comprising p15, p16, P18 and p19) and the KIP/CIP family (p21, p27 and p57) (Sherr *et al.* 1999). The INK family members primarily target Cdk4 and Cdk6. Conversely, the KIP/CIP family members are less specific and broadly interfere with the activities of CDK-Cyclins D/E/A and B complexes (Sherr *et al.* 1999), thus providing an additional regulation level to CDK heterodimers.

3. Post-translational modifications

A third level of CDK regulation is ensured by their phosphorylation within the T-loop, a process required for CDK activation, and within the G-loop that restrains the activity of CDKs to avoid unscheduled DNA replication or entry into M-phase.

▪ *Activatory phosphorylation within the T-loop*

The full activation of CDKs requires their phosphorylation within the T-loop, at T161 for Cdk1 and T160 for Cdk2 (Krek *et al.* 1992, Larochelle *et al.* 2007, Malumbres 2014) (**Fig. 2**). This phosphorylation is controlled by the CDK-Activating Kinase (CAK), an heterodimer comprising Cdk7, Cyclin H and Mat1 (**Fig. 2**). CAK activity remains constant along the cell cycle (Fesquet *et al.* 1993, Poon *et al.* 1993, Solomon *et al.* 1993). The activity of CDK-Cyclin complexes is further restrained by inhibitory phosphorylations.

▪ *Inhibitory phosphorylations within the G-loop*

The activation of CDKs is prevented by the phosphorylation at two residues within the G-loop: T14 and Y15 for Cdk1 and Cdk2, which act by reducing the CDK affinity for their substrates (Malumbres 2014). These phosphorylations are catalysed by the Wee1/Myt1 kinase family and removed by the dual specificity phosphatases family, Cdc25.

– The Wee1/Myt1 kinase family

In Metazoans, the Wee1/Myt1 kinase family comprises Wee1 and Myt1 that phosphorylate and inhibit Cdk1-Cyclin B complexes in interphase. Both Wee1 and Myt1 catalyse the phosphorylation of Cdk1 at Y15 but only Myt1 would phosphorylate CDKs at T14 (Parker *et al.* 1992, Fattaey *et al.* 1997). In *Xenopus* and mammals, two isoforms of Wee1 exists. The maternal or embryonic isoform (unfortunately called Wee1A in *Xenopus* and Wee1B in mammals) is present in oocytes and early embryos and controls the first embryonic cell division (Murakami *et al.* 1998, Nakajo *et al.* 2000, Han *et al.* 2005). The somatic form (Wee1B/Wee2 in *Xenopus* and Wee1A in mammals) is expressed after the MBT (Leise *et al.* 2002, Okamoto *et al.* 2002). Wee1 is a nuclear protein mainly involved in mitotic

cell cycles that prevents active Cdk1-Cyclin B from accumulating in the nucleus during interphase (Parker *et al.* 1992, Heald *et al.* 1993, Baldin *et al.* 1995, Rothblum-Oviatt *et al.* 2001). While Cdk1 is only phosphorylated at Y15 in yeast, Cdk1 can be also phosphorylated at T14 in higher eukaryotes. The kinase responsible for the T14 phosphorylation of Cdk1 was first identified in membrane fractions of *Xenopus* egg extracts and was called Myt1 for Membrane-associated tyrosine-and threonine specific Cdc2 inhibitory kinase (Kornbluth *et al.* 1994, Mueller *et al.* 1995b). Myt1 localizes within the reticulum membranes (Mueller *et al.* 1995b). Besides phosphorylating Cdk1 at T14, Myt1 can also inhibit Cdk1 activation by sequestering Cdk1 in the intracellular membranes (Liu *et al.* 1999, Wells *et al.* 1999). Myt1 is conserved from *C. elegans* to human (Liu *et al.* 1999, Cornwell *et al.* 2002, Okumura *et al.* 2002, Jin *et al.* 2005, Burrows *et al.* 2006). In all these species, Myt1 regulates the G_2/M transition during spermatogenesis and oogenesis, suggesting that Myt1 specifically controls the meiotic divisions of germ cells.

– The dual phosphatases Cdc25

The Cdc25 phosphatase family comprises three isoforms, A, B and C, in mammals while only two isoforms have been identified in *Xenopus* (A and C). These dual phosphatases dephosphorylate Y15 and T14 residues of CDKs, hence activating the CDK-Cyclin complexes. In *Xenopus*, Cdc25C, but not Cdc25A, is essential for promoting mitosis. According the classical view, mammalian Cdc25A regulates the G_1/S transition while Cdc25B and Cdc25C were thought to control the G_2/M transition (Boutros *et al.* 2007). However, more recent evidence suggests that all three isoforms can dephosphorylate Cdk1-Cyclin B complexes and play important roles during the G_1/S and the G_2/M transitions (Boutros *et al.* 2006, Karlsson-Rosenthal *et al.* 2006, Aressy *et al.* 2008). Although an undefined level of functional redundancy exists among the three mammalian Cdc25 phosphatases, a study using siRNA and microscopy in human cells showed that Cdc25A and Cdc25B cooperate for the mitotic entry (Lindqvist *et al.* 2005): Cdc25B activates Cdk1-Cyclin B at centrosomes (Lindqvist *et al.* 2005) and the active Cdk1-Cyclin B complexes are subsequently imported into the nucleus (Jackman *et al.* 2003); while Cdc25A was shown to be involved in chromatin condensation (Mailand *et al.* 2002, Lindqvist *et al.* 2005).

c. Surveillance mechanisms

Importantly, the ordered execution of the cell cycle is coordinated by a series of surveillance mechanisms termed checkpoints (Weinert *et al.* 1988). These mechanisms are not required for cell cycle events themselves but they ensure that each step of the cell cycle is completed correctly before

the cell proceeds to the next phase. Hence, these processes reduce the occurrence of mistakes in the genetic information and segregation, contributing to genome stability. Moreover, they connect the cell cycle progression with the environment, thus allowing the cell to enter in the cell cycle only when the external environment is favourable. Checkpoints are constitutive pathways that consist of at least three components (Murray 1992): a sensor that detects the problem; a signalling pathway composed by mediators/transducers; and a response element in the cell cycle machinery (often a regulator of the CDKs) able to halt cell cycle progression. Three key cell cycle transitions are controlled during the cell cycle (**Fig. 1**): the Start or Restriction point (G_1/S transition) (See chapter: I-A.d.i. "The G_1/S transition"), entry into mitosis (G_2/M transition) (See chapter: I- A.e.ii. "The G_2/M transition") and exit from mitosis (M/G_1 transition) (See chapter: I- A.e.iii.3. "Regulation of APC by SAC"). The Start or restriction point monitors whether the cell can proceed through a new cell cycle in G_1, mostly by sensing the external medium. The entry into mitosis is mostly determined by the completion of DNA replication, while exit from mitosis relies on the Spindle Assembly Checkpoint (SAC) that monitors the quality of the chromosomes alignment on the metaphase plate, necessary to ensure the correct segregation of DNA between the daughter cells.

Cells that meet the size and nutritional requirements can complete the cell cycle unless a specific threat to genome integrity is perceived. One major threat is DNA damage, which is detected by the presence of two DNA structures: Double-Strand Breaks DNA (DSBs) and exposed single-stranded DNA (ssDNA) (**Fig. 3**). These structures are generated during the physiological processes of DNA replication and DNA recombination, but they can be the consequence of the exposure of cells to external insults such as oxidative stresses, ionizing radiations, UV radiations or chemical agents. These checkpoint mechanisms act throughout the cell cycle and arrest the cell cycle progression mainly at G_1/S or G_2/M transitions to allow DNA repair. However, when DNA damages are too extensive, the cell undergoes apoptosis or enters in senescence, a viable state characterised by the definitive incapacity of the cell to divide. The sensors of these DNA damages include the proteins of the PI3 kinase family, ATM (Ataxia telangiectasia mutated) and ATR (Ataxia-telangiectasia-Related). Once activated, these two proteins phosphorylate and activate mediators, Checkpoint kinases 1 and 2 (Chk1 and Chk2), that transduce the signal to the effectors which are part of the cell cycle engine, such as Cdc25 and Wee1. While ATR-Chk1 is traditionally involved in mediating DNA replication and ssDNA checkpoints, ATM-Chk2 is rather implicated in the response to DSBs. However, interconnection between these two networks exists (**Fig. 3**).

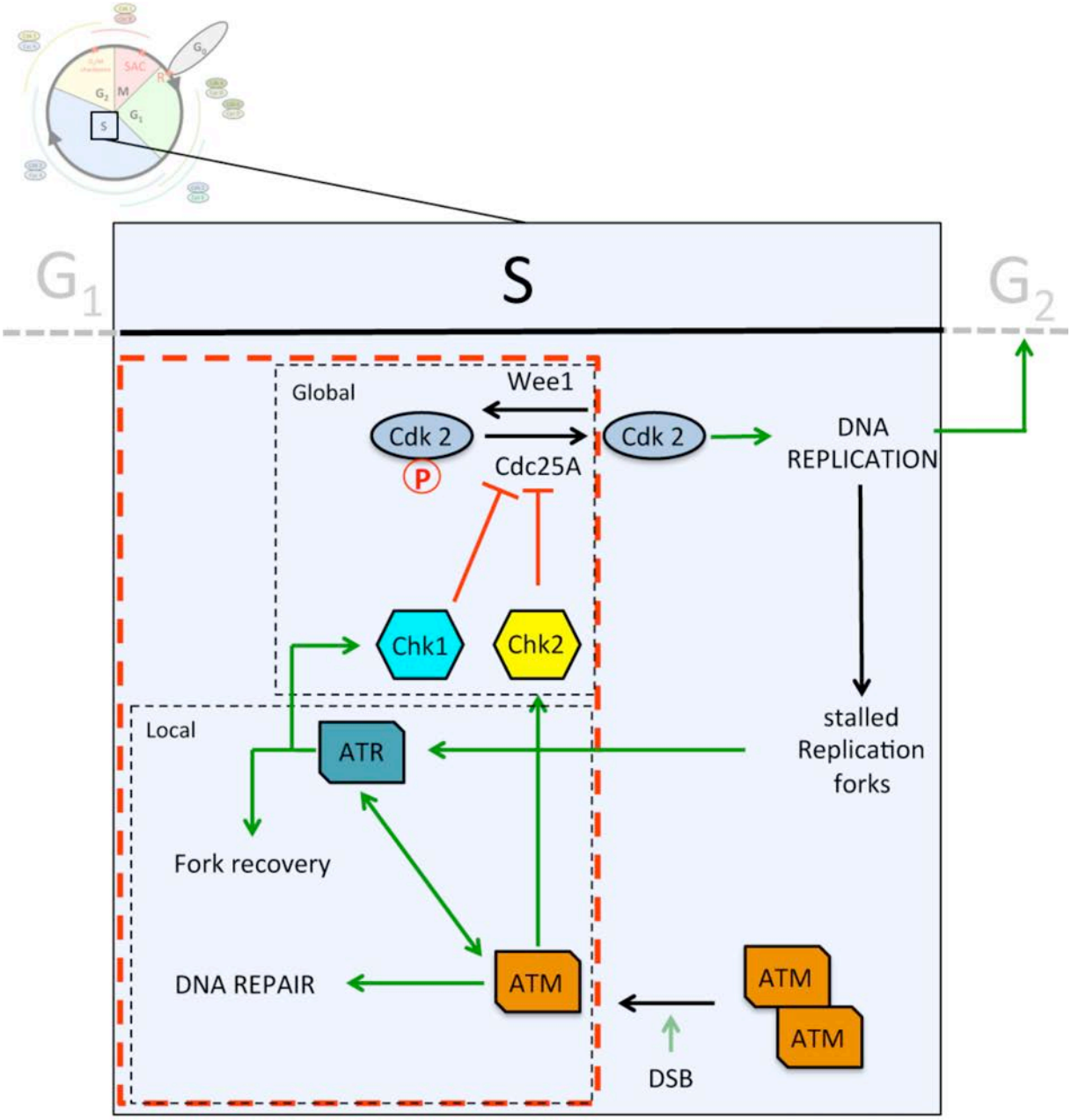

Fig. 3: Checkpoint mechanisms in response to DNA damages during S-phase

In response to double-strand breaks (DSB) or stalled replication forks that generate single-stranded DNA (ssDNA), the sensor kinases ATM and ATR are activated and arrest the cell cycle progression until the damage is repaired. ATM and ATR promote the phosphorylation and the activation of mediators kinases, Chk1 and Chk2, that prevent the activation of Cdc25 and thus the activation of CDKs.

d. Control of S-phase

i. The G_1/S transition

The decision to enter S-phase, which is under the control of the "restriction point", represents a irreversible commitment for the cell to enter and complete mitotic cell cycle. Once this decision is taken, cells become refractory to extracellular signals, being sensible only to their internal environment. The core component of this checkpoint is the retinoblastoma protein (pRB). In the absence of extracellular mitogens, pRB is hypophosphorylated and associates with transcription factors of the E2F family, to repress the expression of E2F-target genes (Dyson 1998) (**Fig. 4**). In response to mitogenic signals, GSK-3β signalling is inhibited, G_1-Cyclin D is stabilized and associates with Cdk4/6 (**Fig. 4**). The activation of these Cdk4/6-Cyclins complexes leads to pRB phosphorylation and causes its dissociation from E2F, which promotes the transcription of genes required for the entry in S-phase, such as S-phase Cyclins (**Fig. 4**).

ii. Molecular control of DNA replication

In Eukaryotes, genome sizes range between 10^7 bp in yeasts to 10^{11} bp in metazoan. The genetic information is distributed across multiple chromosomes. Each of them contains many replicative origins, ensuring a rapid duplication of the eukaryotic genomes. To allow that each chromosome is entirely copied in a reasonable period of time and only once per cell cycle, the initiation of DNA replication from multiple dispersed origins must be coordinated, and thus strictly controlled within the cell cycle. This regulation was enlightened for the first time by performing cell fusion experiments. When G_1 cells are fused with G_2 ones, unreplicated DNA of G_1 cells replicates, but the nuclei of the G_2 cells do not support DNA replication until the hybrid cells have not passed through mitosis (Rao *et al.* 1970). This experiment suggests that G_1 DNA is differently "labelled" compared to the G_2 one, and that the passage through mitosis is necessary to reset the competence to replicate DNA. Nowadays, it is well known that DNA replication is organized into two sequential never-overlapping steps: the "licensing reaction", which confers the competence to replicate (Blow *et al.* 2005), and the origin firing, during which DNA replication starts and origins loose the competence to replicate. Licensing occurs during late M/G_1 and consists in the assembly of pre-Replicative Complexes (Pre-RCs), ending with the loading of the replicative helicase Minichromosome maintenance complex 2-7 (MCM2-7) at replicative origins. During the G_1/S transition, the "origin firing" converts pre-RCs into initiation complexes (ICs), a step that requires S-Cyclin/CDK and DDKs (Cdc7-Dbf4) activities as well as the binding of additional factors. This phase ends with the activation of MCM helicases and the recruitment of the DNA polymerases.

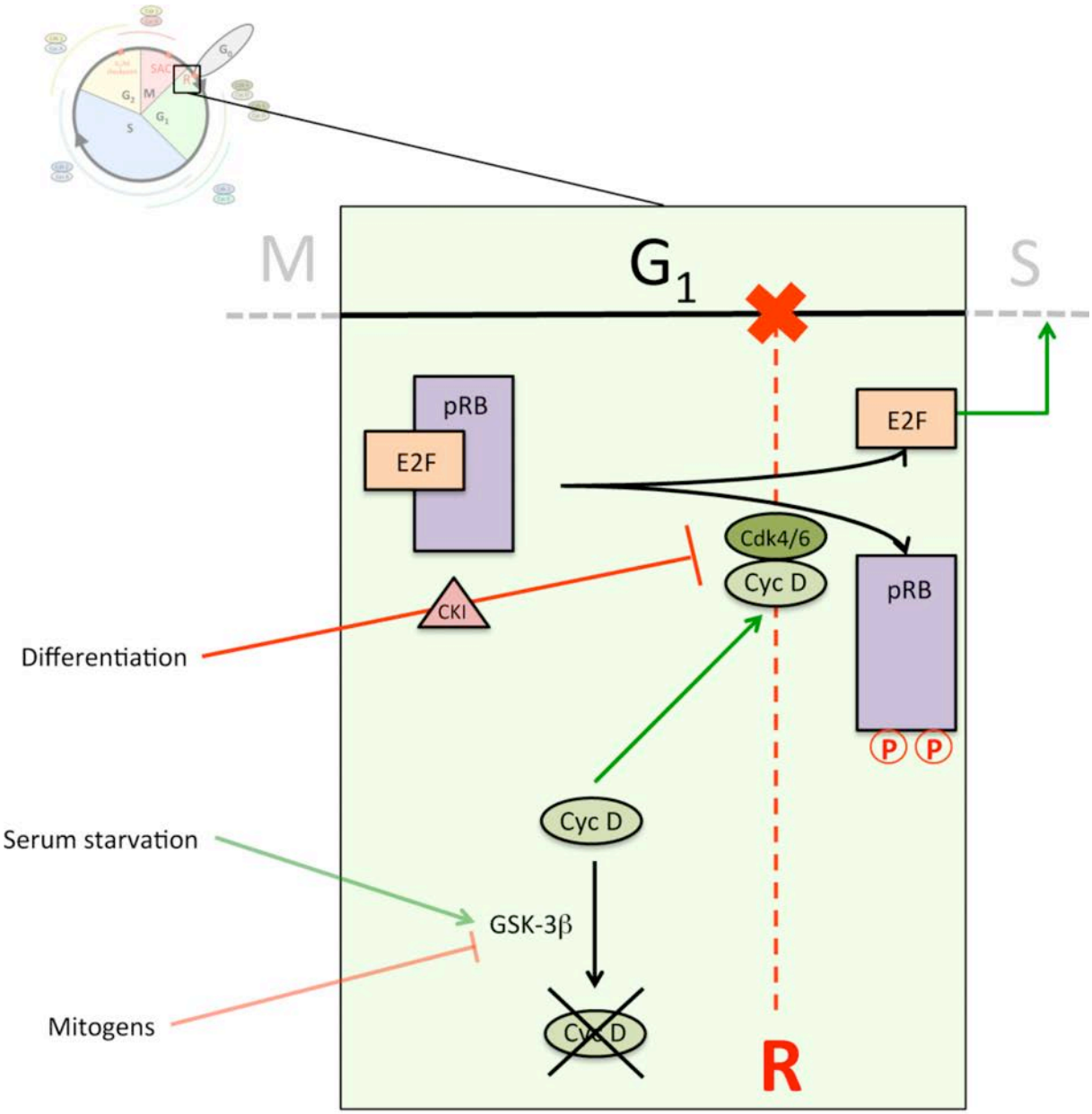

Fig. 4: "The restriction point"

In absence of mitogens, pRB is hypophosphorylated and associates with the transcription factors of the E2F family, repressing their activity. In response to mitogenic signals, GSK-3β signalling is inhibited; G_1-Cyclin D is stabilized and associates with Cdk4/6. Active Cdk4/6-Cyclins complexes then phosphorylate pRB and cause its dissociation from E2F, which in turn promotes the transcription of genes required for the progression in G_1 and the entry in S-phase. During differentiation, the expression of CKI block the activation of Cdk4/6-Cyclin D complexes, thus preventing entry in S-phase.

1. The licensing reaction

The recruitment of MCM2-7 to chromatin requires the stepwise formation of pre-RCs in an ATP-dependent manner on replicative origins. This reaction starts during the M phase with the binding of the Origin Recognition Complex (ORC) to DNA. In budding yeast, this binding requires a conserved sequence of 11-bp or 17-bp, called Autonomously Replicating Sequence (ARS) (Stinchcomb *et al.* 1979), which marks the replication origin on DNA. In Metazoan, ORC binding is not targeted by a clear consensus sequence (DePamphilis 2005). In Eukaryotes, ORC is composed of six subunits (Orc1-6), among them Orc1 that possesses a functional ATPase activity. In G_1, ORC recruits Cdc6, another AAA^+ ATPase, and Cdc10-dependent transcript 1 (Cdt1) that direct the loading of Mcm2-7 complexes onto the DNA. Loaded MCM2-7 consists in an inactive double hexamers that encircles double stranded DNA (Evrin *et al.* 2009, Remus *et al.* 2009a, Remus *et al.* 2009b) (**Fig. 5**).

2. The firing of replicative origins

The inactivation of the APC^{Cdh1}, a ubiquitin ligase responsible on the degradation of Dbf4, together with the progressive accumulation of S-Cyclins lead to the activation of CDKs and Cdc7-Dbf4, the activities of both of them being required for the firing of the origins as well as the replication initiation events (Siddiqui *et al.* 2013) (**Fig. 6**). The origin firing and replication occur continuously during S-phase (Rhind *et al.* 2013). CDK activity promotes Cdc6 phosphorylation and translocation from the nucleus to the cytoplasm (Petersen *et al.* 1999), thus blocking the possibility for fired origins to be licensed again. Cdc7-Dbf4 phosphorylation of MCM2-7 complex promotes the stepwise association of other factors, as the GINS complex, Cdc45, Sld2/3/7, whose phosphorylations by CDKs are required for DNA replication in yeast (Siddiqui *et al.* 2013). As a consequence, MCM2-7 helicases are activated and the DNA polymerase is recruited on the DNA (**Fig. 6**).

3. Elongation and termination of DNA replication

During the elongation step of DNA replication, the MCM helicases move through the DNA and unwind it. The DNA polymerases then load onto the DNA and carry the individual nucleotides to the site of replication by matching new nucleotides to their complementary bases on the parental DNA strand. The leading strand, which runs in the 3' to 5' direction toward the fork, is continuously replicated by DNA polymerases as these enzymes assemble nucleotides from 5' to 3'. As DNA polymerase works, DNA helicase moves down the line to open continuously the replicative fork. Once the polymerase encounters another replicative fork, the polymerase stops and is dissociated from the leading strand.

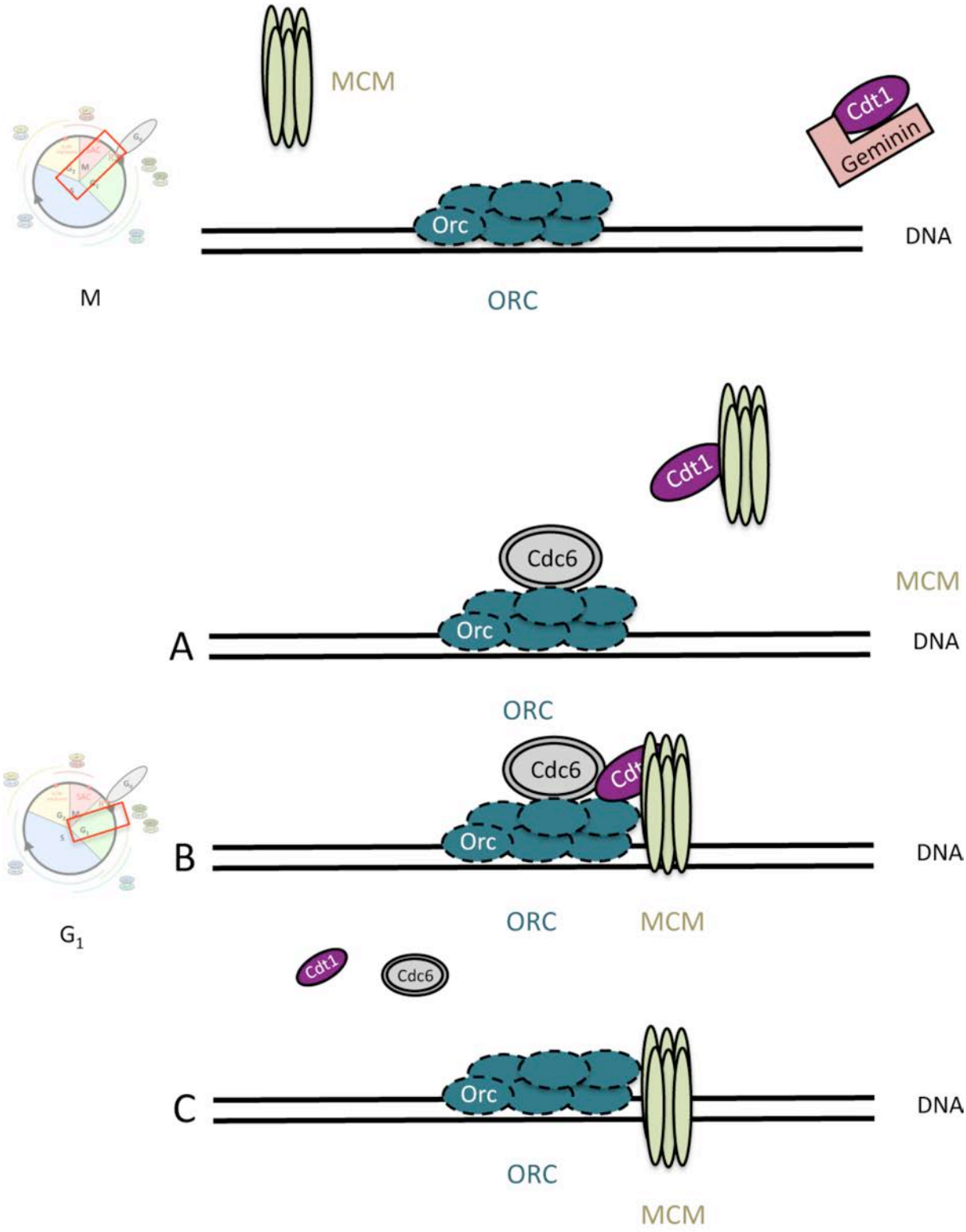

Fig. 5: The licensing of replication origins

In eukaryotes, ORC complex is composed of six subunits (Orc1-6) and recruits Cdc6 and Cdt1 onto the chromatin. Both proteins direct the loading of Mcm2-7 onto the DNA. Loaded MCM2-7 complex remains inactive until the origin firing.

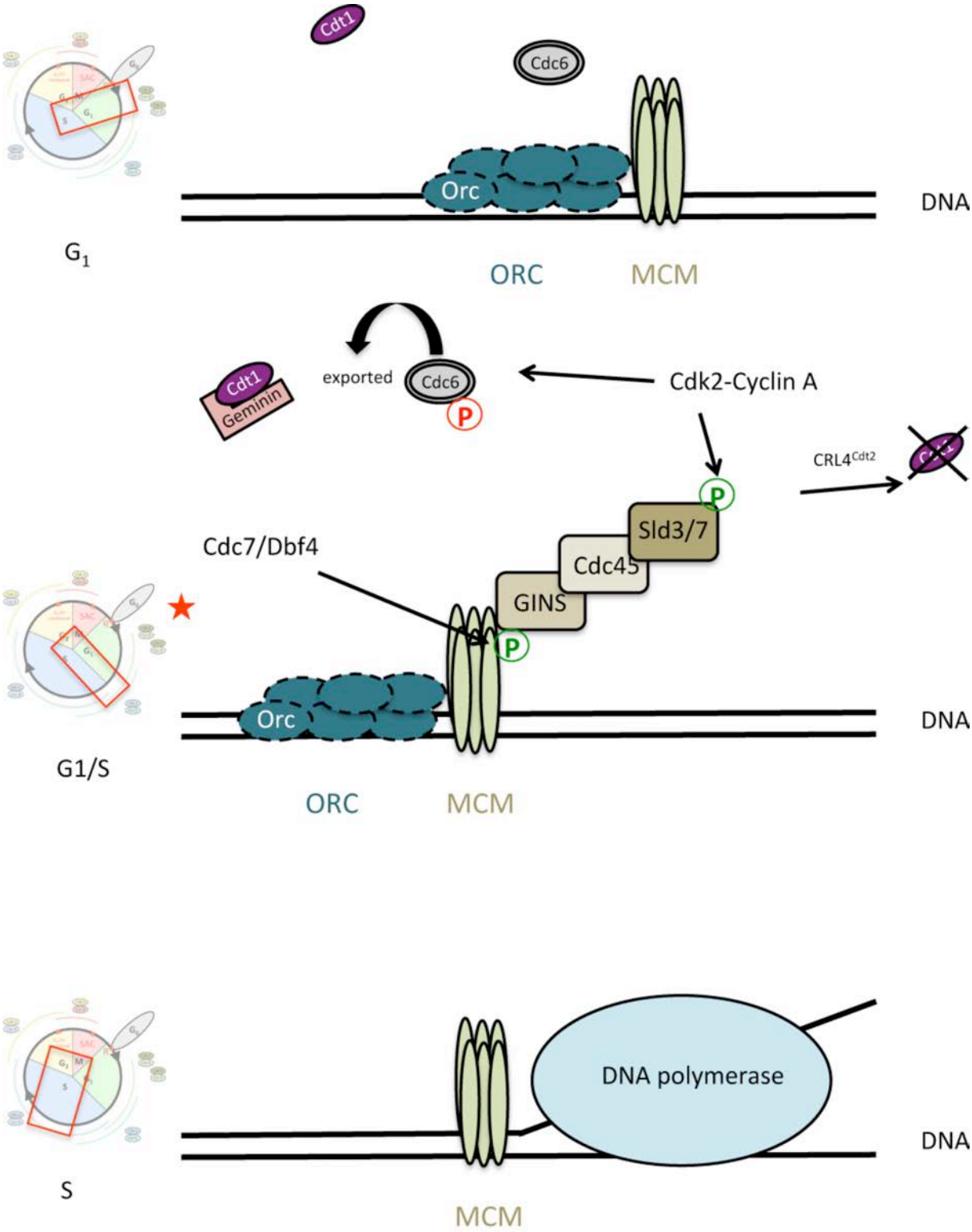

Fig. 6: The firing of replicative origins

The activation of Cdk2-Cyclin A and Cdc7-Dbf4 orchestrate the conversion of Pre-RCs into Ics by promoting the release of some Pre-RC components and the recruitment of new factors. These events activate MCM helicases and lead to the recruitment of the DNA polymerases.

On the lagging strand, DNA polymerases move in the opposite direction toward the replication fork, from 5' to 3' direction. Since DNA polymerases only possess a 5' to 3' activity, the polymerisation of the lagging strand is discontinuous and starts with the action of a RNA polymerase, or Primase, which synthesizes a short RNA polynucleotide (primer). Then, the DNA polymerase extends the 3' end of the primer generating short DNA fragments called Okazaki fragments (**Fig. 7**).

At the end of Okazaki fragment synthesis, DNA polymerase runs into the previous Okazaki fragment and displaces its 5' end containing the RNA primer and a small segment of DNA. This generates a RNA-DNA single strand, which is removed by the flap endonuclease 1 (FEN1) and RNase H. Finally, the gaps between Okazaki fragments and inside the leading strand due to different origins of replication are filled by the DNA polymerase and linked together by a DNA ligase, reconstituting a continuous DNA strand.

iii. Inhibition of DNA re-replication

To ensure that DNA replication occurs only once per cell cycle, licensing and firing of origins are temporally separated by the activity of CDKs that increases during S-phase. CDKs further inhibit the assembly of Pre-RCs once the origin has fired in a way that it cannot be relicensed before the completion of mitosis (**Fig. 8**).

In *S. cerevisiae*, CDKs promote the ubiquitin-dependent degradation of Cdc6 by SCF^{Cdc4} (Drury *et al.* 1997, Elsasser *et al.* 1999, Perkins *et al.* 2001), the nuclear export of MCM-Cdt1 complexes (Labib *et al.* 1999, Nguyen *et al.* 2000) and the inactivation of Orc2 and Orc6 (Nguyen *et al.* 2001, Frigola *et al.* 2013). Since in *S. cerevisiae* many mechanisms to inhibit DNA re-replication are overlapping, the disruption of one mechanism is not sufficient to promote DNA re-replication (Nguyen *et al.* 2000)(**Fig. 8**).

In metazoans, ORC complex is dissembled During S-phase because of the ubiquitination of Orc1 by SCF^{Skp2}, which targets Orc1 for degradation (Mendez *et al.* 2002, Tatsumi *et al.* 2003). Cdc6 is also phosphorylated and when overexpressed, this phosphorylation promotes its export from the nucleus (Petersen *et al.* 1999, Pelizon *et al.* 2000), but a fraction of Cdc6 remains associated to the chromatin (Mendez *et al.* 2000, Alexandrow *et al.* 2004). Furthermore, two additional mechanisms targeting Cdt1 inhibit origin licensing outside G_1 in metazoans. The first one depends on Geminin, which is present from S phase to M-phase and binds Cdt1. The effect of Geminin binding to Cdt1 depends on the ratio between these two components in the complex: when Cdt1 is in excess on Geminin, Cdt1/Geminin complex can participate in the formation of Pre-RC (Lutzmann *et al.* 2006, Xouri *et al.* 2007). However, once DNA replication is started, more Geminin is recruited on chromatin and the

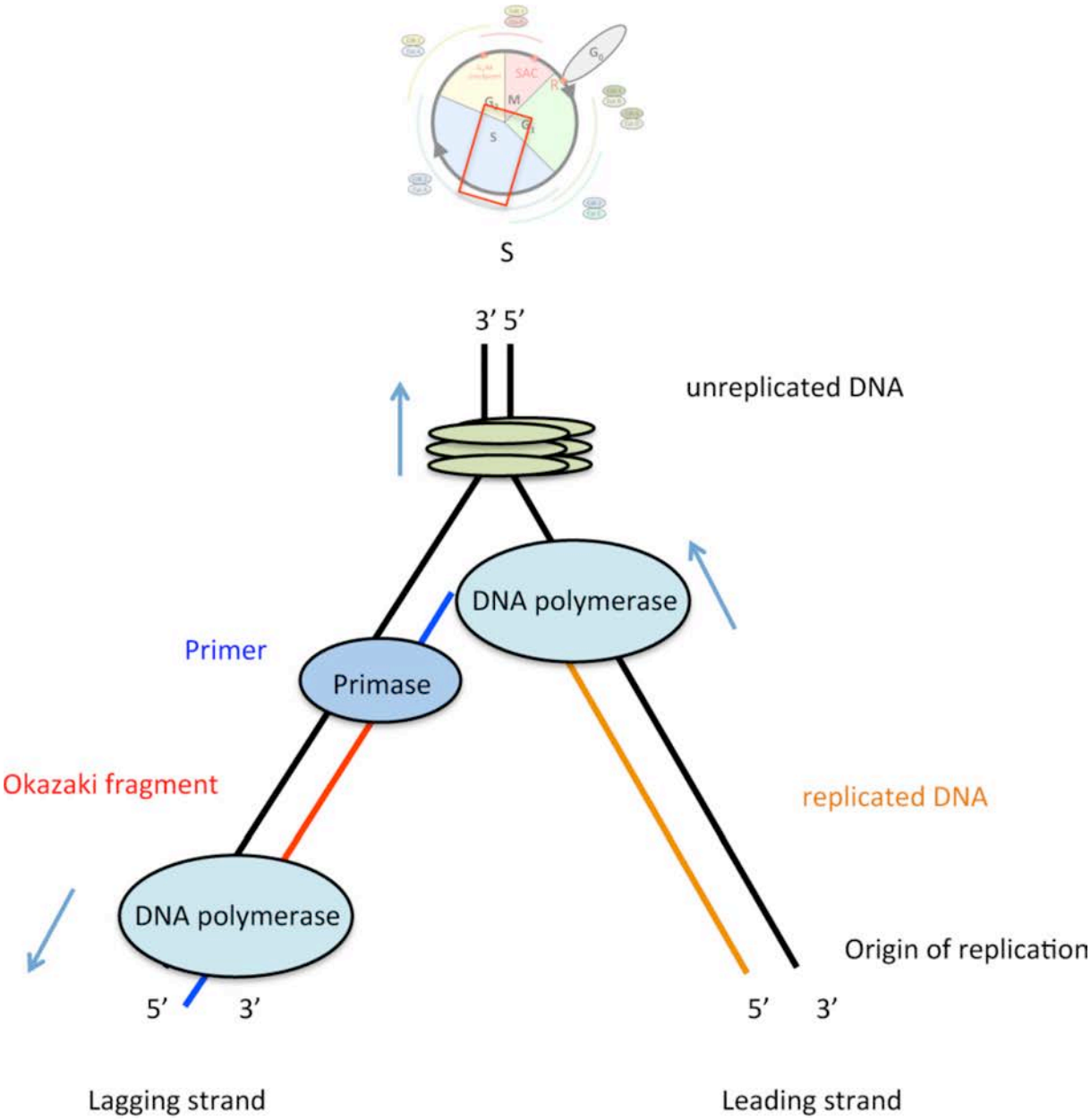

Fig. 7: DNA replication elongation

Two polymerases are present in a replication bubble, each one being associated with one strand of DNA. While the replication of the leading strand can progress continuously, the replication of the lagging strand required the action of a primase, which allows the DNA polymerase to replicate this strand by adding small fragments called Okazaki fragments.

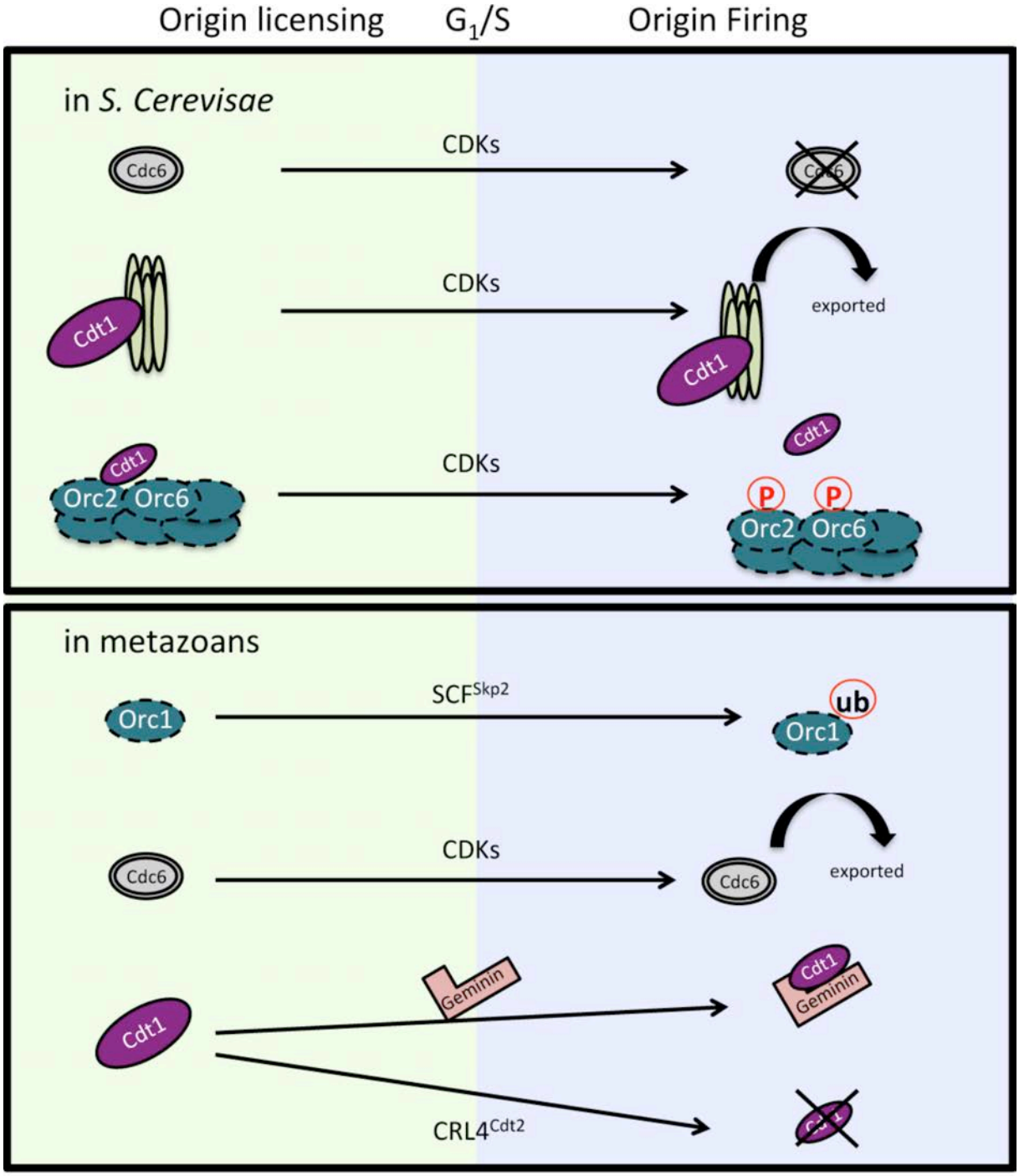

Fig. 8: Inhibition of re-replication

In both *S. cerevisiae* and metazoans, multiple mechanisms are effective to prevent the re-assembly of Pre-RCs once origins has fired during the G_1/S transition. In *S. cerevisiae,* these processes are controlled by the CDK-dependent phosphorylation of Cdc6, Mcm3, Orc2 and Orc6. In metazoans, the SCF^{Skp2}-dependent ubiquitination of Orc1 promotes the disassembly of ORC complex, Cdc6 is phosphorylated by CDKs then exported from the nucleus. Finally, Cdt1 is degraded through its $CRL4^{Cdt2}$-dependent ubiquitination and further inhibited by its interaction with Geminin.

increase of Geminin concentration inhibits the ability of Cdt1/Geminin complex to form Pre-RC (Lutzmann *et al.* 2006). The second mechanism inhibiting Cdt1 is its degradation, which depends on the $CRL4^{Cdt2}$ ubiquitin ligase that recognize Cdt1 PIP-box (Arias *et al.* 2006, Senga *et al.* 2006) (**Fig. 8**). In Xenopus extracts and mammalian cells, the deregulation of Cdt1 provokes re-replication indicating that the mechanisms targeting Cdc6 and ORC are not sufficient to guarantee the inhibition of re-replication (Melixetian *et al.* 2004a, Melixetian *et al.* 2004b, Archambault *et al.* 2005, Arias *et al.* 2005, Li *et al.* 2005, Maiorano *et al.* 2005).

In yeast, Xenopus extracts and human cells, the re-replication process induces the activation of checkpoints mechanisms with the appearance of damaged DNA (Melixetian *et al.* 2004a, Melixetian *et al.* 2004b, Archambault *et al.* 2005, Li *et al.* 2005, Davidson *et al.* 2006). The precise mechanism generating these damages is elusive but may certainly involve fork stalling and collapse (Davidson *et al.* 2006, Neelsen *et al.* 2013).

e. Control of M-phase

The dramatic reorganisation of the cell during mitosis must be coordinated in time and space to support three key events: the entry into mitosis, the sister chromatid segregation and the exit from mitosis.

i. Discovery of Cdk1-Cyclins

In a famous pioneer experiment, Masui and Markert showed that transferring the cytoplasm from a MII-arrested oocyte into a prophase-arrested oocyte of *Rana pipiens* was able to induce meiosis resumption in the recipient oocyte, without hormonal stimulation and independently of the nucleus (Masui *et al.* 1971). The cytoplasmic activity present in MII-arrested oocytes was called “maturation-promoting factor” (MPF). The property of this activity to amplify itself was further demonstrated after serial dilutions and by performing cytoplasmic transfer experiments in the presence of a protein synthesis inhibitor, cycloheximide (CHX), which was unable to prevent meiosis resumption in the recipient oocyte (Masui *et al.* 1971, Wasserman *et al.* 1975). These results demonstrated that a small amount of active MPF present in the cytoplasm of the MII-arrested oocyte was able to activate cytoplasmic inactive-MPF molecules present in prophase oocytes. This process was called “MPF autoamplification”. In the following years, the activity of MPF was shown to be responsible for the entry into mitosis in mammals (Sunkara *et al.* 1979), yeast (Weintraub *et al.* 1982), *Xenopus* (Gerhart *et al.* 1984) and finally in all eukaryotic species tested until now. Hence, MPF being the universal factor promoting entry into M-phase, it was re-baptized “M-phase promoting factor”.

The molecular identification of MPF took 17 years after the discovery of its activity, and was solved by the combination of biochemical and genetic approaches. In starfish oocytes, it was established that an increase in protein phosphorylation was correlated with the oscillations of MPF activity (Doree *et al.* 1983), suggesting the involvement of a kinase during oocyte division. Moreover, it was further discovered that protein synthesis and degradation were as necessary as the kinase burst for the oscillations of MPF activity (Picard *et al.* 1985). This behaviour of MPF activity was reminiscent of a protein recently discovered in sea urchin embryo, whose degradation was necessary to exit from M-phase, the Cyclin (Evans *et al.* 1983). Concomitantly, it was demonstrated that the gene *cdc2* was essential for the G_2/M transition in yeast (Nurse *et al.* 1981). However, it was only following the development of a functional test to easily assay MPF activity, that the molecular composition of MPF was found. This assay was based on the use of *Xenopus* cell-free extracts, generated from activated laid MII-arrested oocytes, supplied with sperm DNA. These extracts were blocked in interphase and were able to mimic entry into mitosis following the addition of purified active MPF, as seen by DNA condensation and the disassembly of the sperm nuclei (Lohka *et al.* 1985). This functional assay was therefore suitable to screen the presence of active MPF in candidate fractions purified from the cytoplasm of MII-oocytes using biochemical approaches such as ammonium sulphate precipitation and chromatography. MPF was shown to be formed of two major proteins of 32 and 45 kDa: Cdk1 (the homolog of the *Cdc2* fission yeast gene), and its associated regulatory subunit, the Cyclin B (Gautier *et al.* 1988, Lohka *et al.* 1988, Gautier *et al.* 1990).

ii. The G_2/M transition

1. The principal player: Cdk1-Cyclin B

Since the expression level of Cdk1 is constant during the cell cycle, the formation of Cdk1-Cyclin B complexes begins during interphase through the increase of Cyclin B synthesis. As Cyclin B accumulates throughout G_2, Cyclin B associates with free Cdk1 molecules, concomitantly with the phosphorylation of Cdk1 at T161 by CAK. However, these newly formed complexes are inactivated by Wee1/Myt1 that phosphorylate Cdk1 at the inhibitory residues T14 and Y15. The counteracting phosphatase Cdc25 is inactive during G_2. Hence, cells do not enter into mitosis. The G_2/M transition is triggered by Cdc25 activation, which promotes the dephosphorylation of Cdk1 at T14 and Y15. Cdk1 then reaches a threshold level of activity (Coudreuse *et al.* 2010) and starts to phosphorylate Cdc25 and Wee1/Myt1, leading to Cdc25 full activation and Wee1/Myt1 complete inhibition, reinforcing and maintaining Cdk1 activity at a high level during the entire M-phase (**Fig. 9**).

The G_2/M transition is tightly regulated by additional mechanisms that coordinate S-phase with mitosis and inhibit unscheduled M-phase entry (**Fig. 9**). These checkpoint pathways, which are

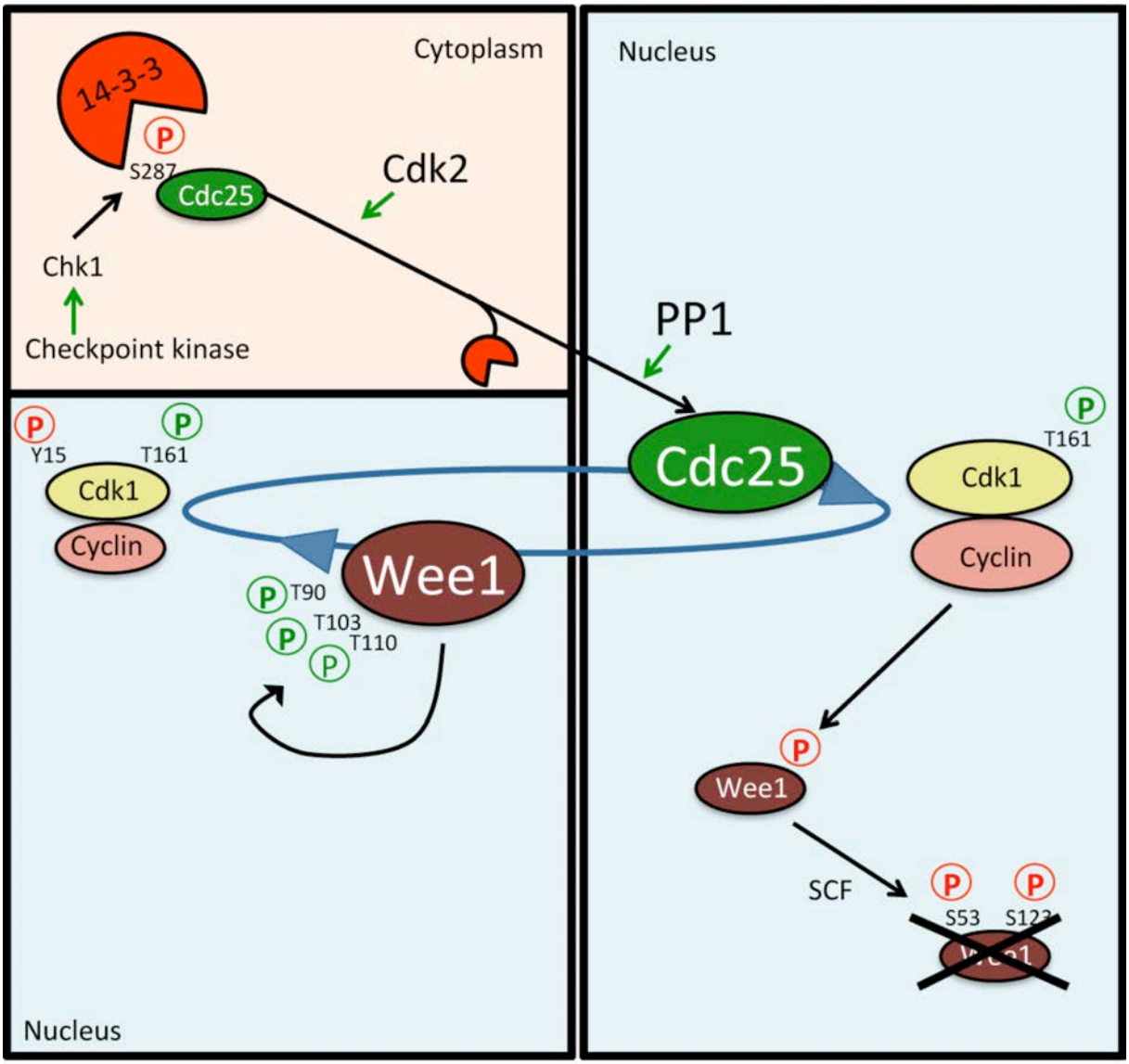

Fig. 9: The control of the G_2/M transition

The G_2/M transition depends on the activation of Cdk1. During G_2, Wee1 is active in the nucleus and Cdc25, mainly cytoplasmic, is inhibited, thus keeping Cdk1 inactive due to its phosphorylation at Y15. The cytoplasmic localisation of Cdc25 is controlled by its phosphorylation at S287, which allows its association to 14.3.3 proteins and sequester the phosphatase in the cytoplasm. During the G_2/M transition, Cdc25 is dephosphorylated by PP1 and activated while Wee1 is inhibited, allowing the progression in M-phase.

activated before the completion of DNA replication or in the presence of DNA damages, shift Wee1/Cdc25 balance in favour of Wee1 to prevent mitosis (reviewed in Langerak *et al.* 2011). These mechanisms directly regulate the activity and the intracellular localization of Wee1/Myt1 and Cdc25.

▪ *Regulation of Wee1*

The regulation of Wee1 involves multiple mechanisms that target its expression level, its activity and its localization. Wee1 levels are regulated transcriptionally (Watanabe *et al.* 1995), translationally (Murakami *et al.* 1998, Charlesworth *et al.* 2000, Nakajo *et al.* 2000) and by ubiquitination and proteolysis (Michael *et al.* 1998). The degradation of Wee1 in M-phase or late G_2 phase is triggered by three SCF-type ubiquitin ligases: SCF^{Tome-1}, $SCF^{\beta\text{-}TrCP1}$, and $SCF^{\beta\text{-}TrCP2}$ (Ayad *et al.* 2003, Watanabe *et al.* 2004). Recognition of *Xenopus* embryonic Wee1 by Tome-1 depends upon the phosphorylation at S38 (Ayad *et al.* 2003), an SP site that is present in *Xenopus* embryonic Wee1A but not in human or mouse embryonic Wee1B. Recognition of somatic Wee1A by β-TrCPs relies on its phosphorylation at two residues present in somatic Wee1 (Wee1B/Wee in *Xenopus* and Wee1A in mammals) proteins but not in embryonic isoforms: S53 (a putative Plk1 phosphorylation site) and S123 (a putative Cdk1 phosphorylation site) (Watanabe *et al.* 2004) (**Fig. 9**).

In addition, Wee1 activity is directly regulated by phosphorylation. During interphase, *Xenopus* Wee1A autophosphorylates at T90, T103 and T110, three poorly conserved residues in its N-terminal regulatory domain (Murakami *et al.* 1999) (**Fig. 9**). Mutation of these sites to phenylalanines decreases Wee1A ability to inhibit *Xenopus* oocyte maturation without decreasing its *in vitro* kinase activity on Cdk1-Cyclin B (Murakami *et al.* 1999). Therefore, this autophosphorylation may regulate the interaction of Wee1A with some factors that modulate its activity or its intracellular localization. During mitosis, the N-terminal non-catalytic domain of Wee1 becomes hyperphosphorylated, causing a shift in its electrophoretic migration, a process that is accompanied by a marked decrease in its activity, which partially depends on Cdk1 activity (Tang *et al.* 1993, McGowan *et al.* 1995, Mueller *et al.* 1995a) (**Fig. 9**). However, the identity of the other kinases that phosphorylate the N-terminal tail of the protein is still controversial. Finally, Wee1 is also regulated by its phosphorylation at S549 (S642 in human), a conserved residue in the C-terminal tail of the protein. The phosphorylation of this site by Chk1 and Akt allows the association of Wee1 with different isoforms of 14-3-3 proteins, producing opposite effects on the cell cycle (Lee *et al.* 2001, Rothblum-Oviatt *et al.* 2001, Katayama *et al.* 2005). Akt phosphorylation at S642 activates human Wee1 binding to 14-3-3θ, which decreases Wee1 activity and sequesters the protein in the cytoplasm, therefore inducing mitosis (Katayama *et al.* 2005). On the other hand, Chk1 promotes Wee1 binding to 14-3-3β, increasing Wee1 activity and stability, and inhibiting the G_2/M transition (Lee *et al.* 2001, Rothblum-Oviatt *et al.* 2001).

Phosphorylation is certainly an important way of negatively regulating Wee1 function during M-phase, in particularly in systems like *Xenopus* embryos, where the overall abundance of Wee1A varies little between interphase and M-phase (Murakami *et al.* 1998).

- ***Regulation of Cdc25 phosphatase***

As for Wee1, the activity of Cdc25C is regulated by its phosphorylation that modulates its activity and controls its intracellular localization, which depends on one nuclear localization signal (NLS) and two nuclear export signals (NES).

In interphase, the phosphatase activity of Cdc25C is low (Kumagai *et al.* 1992). The protein is localized in the cytoplasm due to its phosphorylation at S216 (S287 in *Xenopus*), which promotes its interaction with 14.3.3 (Furnari *et al.* 1997, Peng *et al.* 1997, Kumagai *et al.* 1998a, Kumagai *et al.* 1998b, Zeng *et al.* 1998, Furnari *et al.* 1999, Kumagai *et al.* 1999, Yang *et al.* 1999, Graves *et al.* 2001, Lopez-Girona *et al.* 2001). Following DNA damage or DNA replication-activated checkpoints, the kinases Chk1 and Chk2 are activated and phosphorylate Cdc25 at S287, sequestering Cdc25C within the cytoplasm and arresting the cell cycle progression in G2 in yeast, *Xenopus* and human (Furnari *et al.* 1997, Peng *et al.* 1997, Sanchez *et al.* 1997, Zeng *et al.* 1998, Furnari *et al.* 1999, Boutros *et al.* 2007) (**Fig. 9**). Moreover, other kinases can phosphorylate Cdc25C at S287, among them are PKA (Duckworth *et al.* 2002), C-TAK (Peng *et al.* 1998) and CAM Kinase II (Hutchins *et al.* 2003). Since the activation of these kinases occurs independently of checkpoint mechanisms, this further suggests that additional mechanisms restrain Cdc25C activation.

During the G_2/M transition, Cdc25C must be dissociated from 14-3-3 proteins to be imported in the nucleus and activated. The first step required for the activation of Cdc25C depends on its phosphorylation at T138 (T130 in human), which is controlled by the balance of activities between Cdk2 and the phosphatase PP2A-B56δ (Rosenblatt *et al.* 1992, Izumi *et al.* 1993, Rempel *et al.* 1995, Guadagno *et al.* 1996, Margolis *et al.* 2003, Margolis *et al.* 2006a, Margolis *et al.* 2006b) (**Fig. 9**). Once T138 is phosphorylated, 14.3.3 is released from Cdc25C. This allows the binding of another phosphatase, PP1, which in turn promotes the dephosphorylation of Cdc25C at the inhibitory residue S287 (Margolis *et al.* 2003, Margolis *et al.* 2006b) (**Fig. 9**).

In mammals, it has been further proposed that the T130 phosphorylation of Cdc25C creates a docking site for the kinase Plk1 that, in turn, phosphorylates Cdc25C at S198 (Elia *et al.* 2003a, Elia *et al.* 2003b). This residue being localized within the NES of Cdc25C, this phosphorylation would promote the nuclear import of Cdc25C during the mitotic prophase, before the nuclear envelope breakdowns (Toyoshima-Morimoto *et al.* 2001). Beside Cdk2 and Plk1, the last kinase that efficiently phosphorylates Cdc25C is Cdk1 itself, thus creating a positive feedback loop that induces the full

activation of Cdc25C (Izumi *et al.* 1993). One site in particular, S285 in *Xenopus* (S214 in human), is required for the full activation of Cdc25C and its mutation into alanine dramatically decreases the ability of Cdc25C to promote M-phase entry in human and *Xenopus* (Bulavin *et al.* 2003a, Bulavin *et al.* 2003b, Margolis *et al.* 2006b). This phosphorylation stimulates directly the activity of Cdc25C as well as its interaction with PP1, thus keeping Cdc25C dephosphorylated at S287 (Bulavin *et al.* 2003a, Bulavin *et al.* 2003b, Margolis *et al.* 2006b). This last feedback mechanism, between Cdk1 and Cdc25C, maintains the phosphatase activity of Cdc25C at a high level during M-phase (**Fig. 9**).

2. Role of protein phosphatases

M-phase obviously depends on the activation of numerous kinases under the direct or indirect control of Cdk1-Cyclin B. It had been suspected that the opposite activities of phosphatases could be regulated as well. This was indeed revealed by genetic screens in fission yeast and small interfering RNA screens in *Drosophila* (Kinoshita *et al.* 1990, Chen *et al.* 2007). These two screens identified PP2A and PP1 as major actors for mitosis progression, particularly in anaphase and during mitotic exit. Still, it is the use of *Xenopus* cycling extracts that clearly demonstrated the role of PP2A during mitotic entry (Mochida *et al.* 2009). PP2A phosphatase is a multimeric complex composed of a catalytic subunit (C-subunit), a non-catalytic scaffolding subunit (A-subunit) and a B-subunit that confers the substrate specificity. It was shown that the activity of one specific PP2A holoenzyme, PP2A-B55δ, is regulated in cycling extracts: being high in interphase and low during mitosis (Mochida *et al.* 2009). In addition, the immunodepletion of PP2A-B55δ in interphase extracts accelerates the entry into mitosis and arrests the extracts at this stage, whereas removing it during M-phase strongly delays the exit from mitosis (Mochida *et al.* 2009). Furthermore, adding recombinant PP2A-B55δ in cycling extracts efficiently inhibits entry into M-Phase (Mochida *et al.* 2009). These results demonstrate for the first time that the activation of Cdk1-Cyclin B is not sufficient for M-phase entry and progression and that a member of the PP2A family, PP2A-B55δ, needs to be simultaneously inactivated.

This inhibition of PP2A-B55δ depends on one specific kinase, Greatwall (Gwl), which was identified by a screen aiming at finding new regulators of mitosis in *Drosophila* (Blake-Hodek *et al.* 2012). Gwl is activated upon M-phase entry by a Cdk1-dependent phosphorylation at T193 and T206 and by autophosphorylation at S833 (Blake-Hodek *et al.* 2012, Lorca *et al.* 2013) (**Fig. 10**). When Gwl is immunodepleted from *Xenopus* mitotic extracts, Cdk1 is quickly inactivated in the presence or in the absence of Wee1 and Myt1 kinases (Yu *et al.* 2006, Vigneron *et al.* 2009). These results indicate that

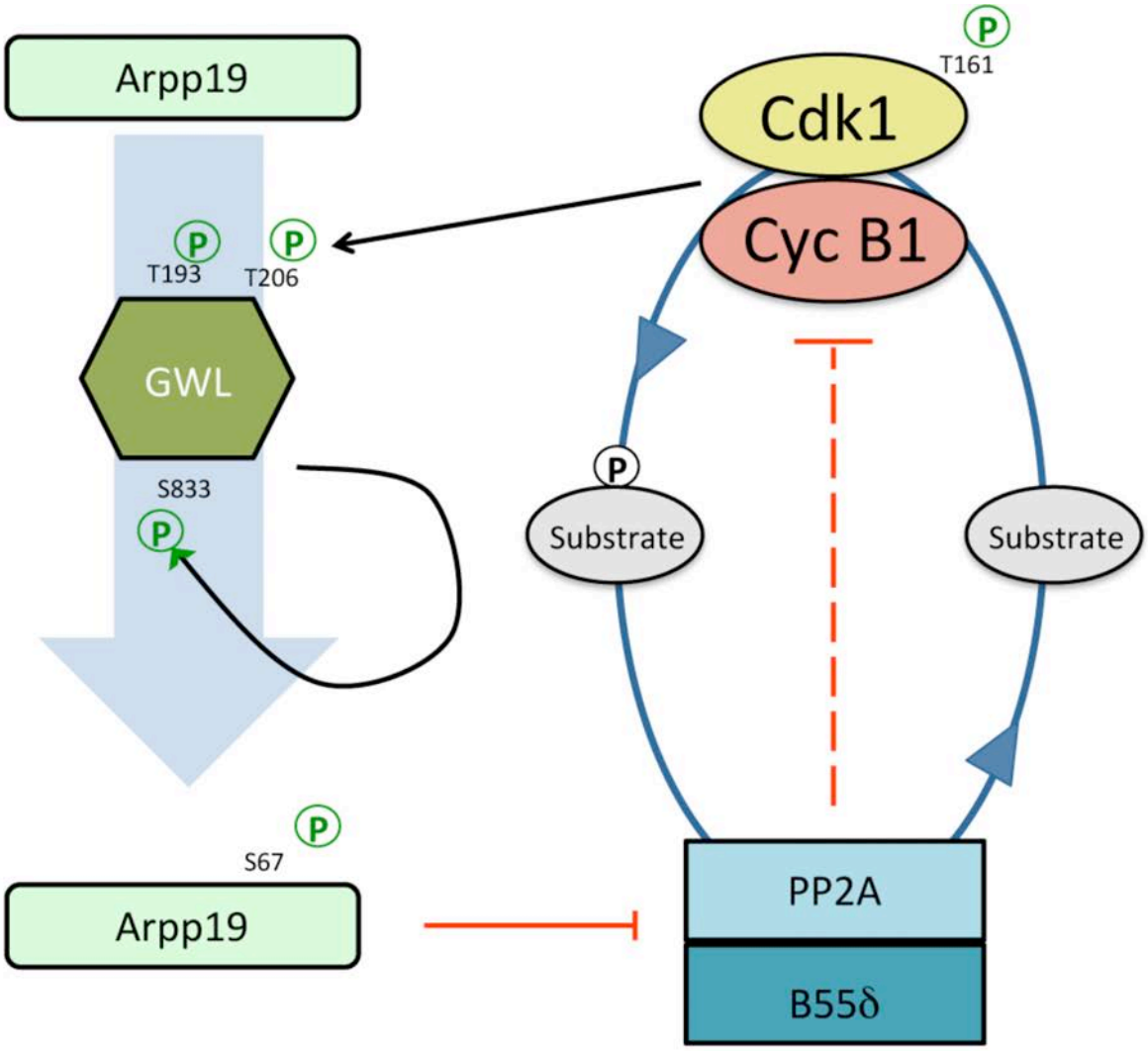

Fig. 10: The Gwl/Arpp19/PP2A module

Cdk1 activation is not sufficient by itself to promote M-phase entry and the inhibition of PP2A-B55δ phosphatase is further required for the phosphorylation of mitotic substrates. The inhibition of PP2A-B55δ depends on the Cdk1-dependent activation of Gwl that phosphorylates Arpp19 at S67 and converts this protein in a potent inhibitor of PP2A-B55δ by direct binding.

Gwl is required to maintain a mitotic state even in the presence of active Cdk1 and controls the phosphorylation status of Cdk1 substrates. Since the establishment of M-phase, after Gwl depletion, is rescued by either immunodepleting PP2A or inhibiting PP2A with okadaic acid (Vigneron *et al.* 2009), it was concluded that Gwl controls directly or indirectly PP2A activity. This process is indeed mediated by a small protein of the α-endosulfine family, ARPP19/ENSA, which was identified by mass spectrometry, thanks to its ability to be phosphorylated by Gwl (Gharbi-Ayachi *et al.* 2010, Mochida *et al.* 2010). ARPP19/ENSA is phosphorylated by Gwl at a serine (S67 in *Xenopus*) present in a specific highly conserved motif (RGD**S**). The phosphorylation of ARPP19 at S67 by Gwl converts Arpp19 into a potent inhibitor of PP2A-B55δ that acts by direct binding (Gharbi-Ayachi *et al.* 2010, Mochida *et al.* 2010) (**Fig. 10**). Phosphorylated ARPP19/ENSA acts as a competitive substrate. It is slowly dephosphorylated by PP2A-B55δ, allowing PP2A to be reactivated at the time when Cdk1-Cyclin B and Gwl are inactivated (Williams *et al.* 2014). This Gwl-ARPP19 module is conserved from yeast to man, with differences. In human cells, as in *Xenopus*, Mastl (Gwl homologue) is necessary to to enter mitosis and thereafter all over mitosis, and its complete depletion arrests the cells in G_2 (Burgess *et al.* 2010). In yeast, PP2A promotes M-phase entry by antagonizing the inhibitory effect of Swe1, the homolog of Wee1, on Cdk1. The pro-mitosis role of Gwl/ARPP19 module is conserved, because Igo1/2 (Arpp19 homologs) contribute to localize and maintain high PP2A activity, which in this system promotes mitotic entry (Juanes *et al.* 2013). In addition to PP2A, PP1 phosphatase has been shown to play an important role in entry of M-phase, although the control exerted by PP1 is either positive or negative according the model systems. Importantly, free monomeric PP1 catalytic subunit has a broad substrate specificity and for this reason it was initially considered as a not specific housekeeping phosphatase. This view has changed by the discovery that PP1-intercating proteins (PIPs) are able to finely modulate PP1 activity. Therefore, PP1 interaction with different PIPs certainly contributes to substrate specificity and determines how a particular PP1-PIP complex is regulated. Around 180 mammal proteins are known to interact with PP1, between them are present: PP1 substrates, proteins necessary for PP1 intracellular localization and proteins modulating or inhibiting its phosphatase activity (Reviewed in Bollen *et al.* 2010).

During the G_2/M transition, PP1 is involved in the activation of Cdc25, promoting its dephosphorylation at S287. During metaphase, PP1 localized at the kinetochores dephosphorylates Aurora B substrates, stabilizing microtubules attachment at the kinetochore and promoting Spindle Assembly Checkpoint (SAC) silencing (Liu *et al.* 2010, Meadows *et al.* 2011) (See chapter I-A.e.iii.3. "Regulation of the APC by SAC") (**Fig. 12**). PP1 involvement in these two processes suggests that the phosphatase need to be activated for the entry and the execution of M-phase. Alternatively, PP1 phosphatase counteracts the activity of NIMA-related kinase 2 (Nek2), whose activation contributes

to the separation of the duplicated centrosomes to create two independent microtubule organizing centres prefiguring the two poles of the mitotic spindle (Ghosh *et al.* 1998, Helps *et al.* 2000, Meraldi *et al.* 2001). In addition, it was also been shown that Cdk1 directly phosphorylates PP1 at T320, inhibiting its phosphatase activity (Dohadwala *et al.* 1994, Kwon *et al.* 1997). Finally, both PP2A and PP1 are required for mitotic exit, catalysing the dephosphorylation of the mitotic substrates (Mochida *et al.* 2009, Wu *et al.* 2009, Schmitz *et al.* 2010). These last results suggest that PP1 is inhibited during M-phase, to prevent early dephosphorylation of mitotic substrates, and activated only at anaphase onset. The high combinatory number of the PP1-PIPs complexes could explain the widely differentiated, and sometimes contradictory, roles of PP1 during mitosis, suggesting that probably some PP1-PIP complexes need to be activated and others inhibited during the entry and the execution of the M-phase. Therefore, much work has still to be done to clarify the specific roles of PP1 in the cell division, each of them probably depending on specific subunit of PP1 and of a tight control of its intracellular localization.

iii. The exit from M-phase

Once the cell has passed the metaphase, its progression through division is mainly regulated by the APC/C degradation system, which targets two proteins: the securin, a specific inhibitor of a protease, called separase, that is responsible for the cleavage of the cohesin rings that hold the sister chromatids together and the Cyclin B, leading to the irreversible inactivation of Cdk1 (Reviewed in Harper *et al.* 2002, Peters 2002) (**Fig. 11**). However, inactivating Cdk1 is not sufficient for the return in interphase and the activation of PP2A-B55δ and PP1 phosphatase is further required to hydrolyse the phosphates incorporated within mitotic Cdk1 substrates.

1. The ubiquitination of Cyclins

The degradation of mitotic Cyclins is mediated by two particular motifs within the N-terminus of Cyclin B: the Destruction Box (D-Box), which is necessary and sufficient for the degradation of the protein in *Xenopus* egg extracts and the KEN-box (Evans *et al.* 1983, Murray *et al.* 1989, Glotzer *et al.* 1991). These sequences correspond to a two-part degradation signal or degron, which consists in a disordered region within the substrate where a polyubiquitin chains can be reversibly attached. This reaction consists in the formation of an iso-peptide binding between the ubiquitin and a lysine residue on the target protein. Since ubiquitin contains some lysine residues (Lys 63, Lys 11, Lys 48), an ubiquitin chain can be polymerised. This polyubiquitination chain is attached by the sequential

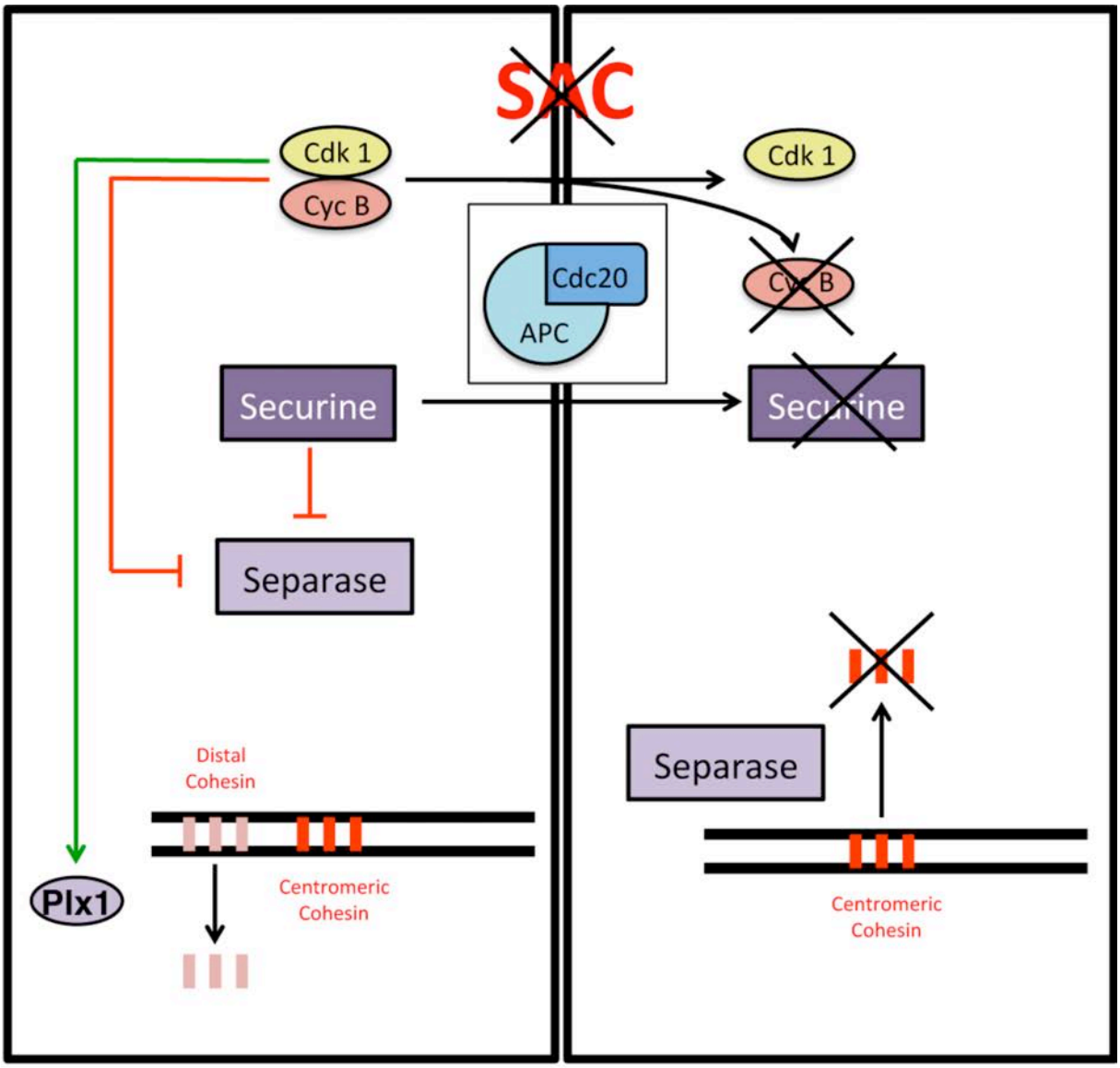

Fig. 11: The Metaphase to Anaphase transition

During the early phases of M-phase, Plx1 is activated and phosphorylates distal cohesin, removing them from the arm of chromosomes. Following the correct bipolar attachment of the kinetochores, the Spindle Assembly Checkpoint or SAC is silenced and APC^{Cdc20} is activated. This complex controls the ubiquitination and the degradation of Cyclin B, causing Cdk1 inactivation, and of Securin, promoting the activation of the protease Separase that is responsible for cutting the cohesins. The chromosomes can then segragate.

action of three enzymes: a ubiquitin activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligase (E3). While the mono-ubiquitination of a protein controls its activity, its localization and its interaction with partners, a chain of at least 4 ubiquitins is required for the proper recognition of the substrate by the proteasome. This process involves the 19S subunit of the proteasome that unfolds the protein and directs the substrate to the cavity of the 20S subunit responsible for the protein degradation. The activity of the proteasome is constant during cell cycle, whereas the ubiquitination of the substrate that has to be degraded by the proteasome is highly controlled, mainly by the E3 regulation.

2. The Anaphase promoting Complex (APC)

During mitosis, the main ubiquitin ligase responsible for Cyclin B ubiquitination is the APC/C, which is a cullin-RING finger E3 ubiquitin ligase composed of 15-17 subunits depending on the species. These subunits are organized in two sub-complexes bridged together by APC1. The first sub-complex contains APC3 and APC10 that respectively bind two co-activators, Cdc20 (Fizzy) and Cdh1 (Fizzy-related protein), which confer the substrate specificity acting as a Degron receptor. The second sub-complex contains the catalytic subunit, APC2, and the subunit able to contact E2, APC11.

During metaphase, the Cdk1-dependent phosphorylation of several APC core subunits as well as Cdh1 promotes the recruitment of Cdc20 and simultaneously prevents Cdh1 from interacting with APC10. APC^{Cdc20} is therefore the only complex active at the end of metaphase and coordinates the inactivation of Cdk1 and the separation of sister chromatids (**Fig. 11**). Besides catalysing Cyclin B degradation, the proteasome possesses also a "non proteolytic activity" able to dissociate polyubiquitinated-Cyclin B from Cdk1 (Nishiyama *et al.* 2000). This process also depends on APC and contributes to Cdk1 inactivation (Chesnel *et al.* 2006). Altogether, these experiments enlighten the importance of the APC/C as the main regulator of M/G_1 transition.

3. Regulation of the APC by SAC

In mitosis, APC/C is activated coincidently with the Nuclear Envelope Break Down (NEBD) in metazoan cells (den Elzen *et al.* 2001, Geley *et al.* 2001, Wolthuis *et al.* 2008). The activation of APC/C is tightly regulated by the Spindle Assembly Checkpoint (SAC), which monitors the correct and bipolar attachment of the kinetochores to the metaphase plate and prevents the APC/C from recognizing its substrates unless this step is not correctly achieved (Hoyt *et al.* 1991, Li *et al.* 1991) (Reviewed in Lara-Gonzalez *et al.* 2012). In prometaphase, the major SAC proteins Mad2, BubR1/Mad3 and Bub3 form the Mitotic Checkpoint Complex (MCC) at kinetochores. Kinetochore-associated MCC binds and sequesters Cdc20 at kinetochores, thus preventing APC activation and the

consecutive proteolysis of the mitotic Cyclins and securin (Reviewed in Lara-Gonzalez *et al.* 2012). At metaphase, when sister kinetochores are fully attached to kinetochore microtubules and correctly bioriented, MCC is dissociated from the kinetochores, Cdc20 is released and hence activates APC (Musacchio *et al.* 2007) (**Fig. 12**).

4. The metaphase to anaphase transition

Once SAC is turned off, the APC/C starts to degrade Cyclin B and securin, which are both inhibitors of the separase (Stemmann *et al.* 2001, Gorr *et al.* 2005, Holland *et al.* 2006) (**Fig. 11**). Separase is therefore activated and cleaves the Scc1 subunit of the cohesin complex that holds together the two sister chromatids. In animal cells, most of the cohesin complexes located on chromosome arms are removed as a consequence of phosphorylation by the kinase Plk1 (Hauf *et al.* 2005) (**Fig. 11**). However, the complexes located at the centromeres are not released, being protected by the Shugoshin protein that recruits the PP2A-B56 phosphatase and prevents the phosphorylation by Plk1 (Kitajima *et al.* 2006, Riedel *et al.* 2006, Tang *et al.* 2006). These centromere-located cohesin complexes are eventually degraded by separase, allowing sister chromatids separation (**Fig. 11**). After anaphase, the cell exits from mitosis due to the degradation of Cyclin B that keeps Cdk1 activity below the level required to maintain the mitotic state. The cell executes cytokinesis and returns to interphase by re-shaping its intracellular structure. Part of this process relies on the APC/C that degrades Plk1 and Aurora kinases (Lindon *et al.* 2004, Floyd *et al.* 2008). Furthermore, PP1 and PP2A are required for controlling anaphase and mitosis exit (Kinoshita *et al.* 1990, Chen *et al.* 2007, Wurzenberger *et al.* 2012). The activity of PP1 increases in anaphase (Wu *et al.* 2009) and targets multiple substrates that coordinate processes such as chromosomes decondensation (Vagnarelli *et al.* 2011) and nuclear envelope reassembly (Steen *et al.* 2000). The drop in Cdk1-Cyclin B allows the inactivation of Greatwall, which leads to the dephosphorylation of ARPP19/ENSA at S67, reactivating PP2A-B55δ (Cundell *et al.* 2013, Williams *et al.* 2014). This specific PP2A isoform actively dephosphorylates all the Cdk1 substrates. As a consequence, the cell exits from mitosis and enters in G_1. The low level of Cdk1 activity leads to the formation of APC^{Cdh1} that, in turn, degrades Cdc20, as well as the Geminin, an inhibitor of Cdt1, allowing the cell to form the pre-RCs. The cell will remain arrested in G_1 until the conditions are favourable to be engaged in another cell cycle.

B. The meiotic division

Meiosis is a particular mode of cellular division that generates haploid cells from a mother diploid cell. This process is required for sexual reproduction in eukaryotes. Sexual reproduction has the

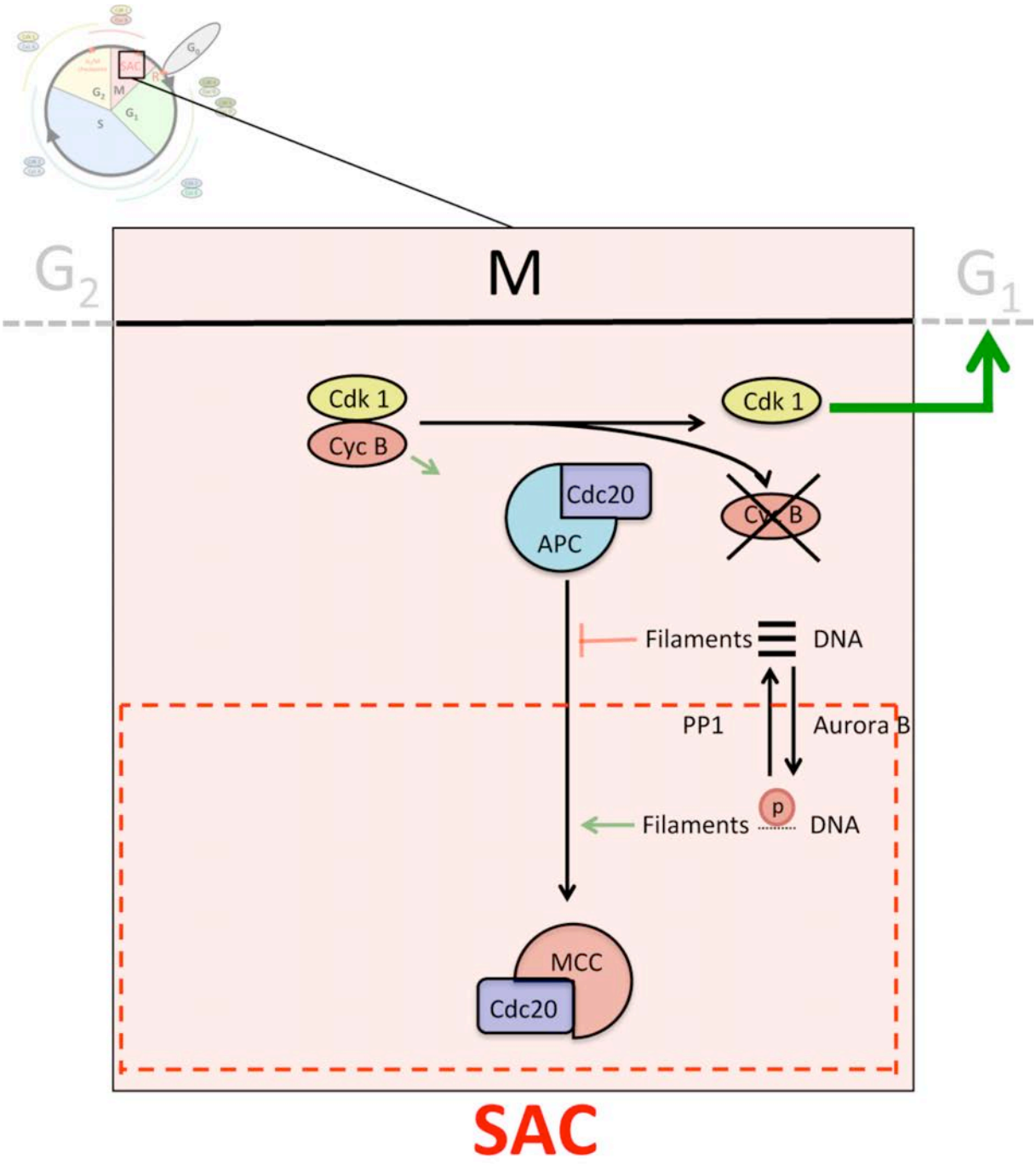

Fig. 12: The Spindle Assembly Checkpoint (SAC)

The SAC monitors the correct and bipolar attachment of kinetochores to the spindle. SAC activation/silencing depends on the opposite activity of Aurora B kinase and PP1, which are regulated by the tension caused by the bipolar attachments. When chromosomes are not well attached, Aurora B phosphorylates outer kinetochore proteins, destabilizing their attachment and promoting the assembly of the Mitotic Checkpoint Complex (MCC). Once the chromosome are bipolary attached (and under tension), Aurora B is removed from centromers and PP1 dephosphorylates the kinetochore proteins, leading to MCC inactivation.

potentiality to generate diversity by mixing the genetic information coming from two individuals thanks to the presence of three events: recombination (crossing over), chromosome segregation and fertilization. The two first ones occur during meiosis.

The meiotic divisions consist in two cellular divisions (meiosis I and meiosis II) with no intervening S-phase, leading to the production of four haploid cells. Their fate depends on the specie and the sex of the individual. In the animal kingdom, the 4 spermatozoa are viable whereas the female progenitors undergo two asymmetrical divisions leading to the production of one oocyte and 2 unviable cells, referred as polar bodies 1 and 2 (PB1 and PB2). I will focus on female meiotic divisions that are the paradigm used in my PhD work.

a. Characteristics of the female meiotic division

Meiotic divisions evolved from mitotic divisions. Hence many aspects of both processes are similar, except those specific of meiosis that are critical for the accurate reduction of the parental genome (**Fig. 13**).

i. Two natural arrests during the meiotic cell division

Female meiotic divisions arrest naturally at two specific steps. These two arrests are physiological and are therefore independent of any checkpoint mechanisms.

The first arrest is universal in all animal species and occurs in prophase of the first meiotic division (prophase I) (See chapter I-B.c. "the arrest in prophase") This arrest lasts from months to years depending on the species and is required for the accumulation of mRNAs and proteins necessary for the development of the future embryo. Regarding the biochemistry of the cell cycle, this arrest at prophase I is assimilated to a G_2 arrest, and its release is comparable to a G_2/M transition since the regulation of Cdk1 operating during this period is similar to the ones described for mitosis.

The secondary arrest occurs during the second meiotic division and concerns laid oocytes. It provides a time window for awaiting fertilization. This secondary arrest prevents the oocyte to undergo cell divisions and development in the absence of fertilization, a process called parthenogenesis. It corresponds to the stage at which the oocyte is fertilized. The step of this arrest depends on the species and can occur either in metaphase of the first meiotic division or MI (some arthropods, some molluscs), in metaphase of the second meiotic division or MII (all Vertebrates) (See chapter I-B.f. "The CSF arrest in vertebrates"), or at the pronucleus stage once the second meiotic division is completed (a G_1 arrest) in many Invertebrates (for examples as model systems: sea urchins and starfish). In some cases, the first arrest at prophase I corresponds also to the second arrest: it applies

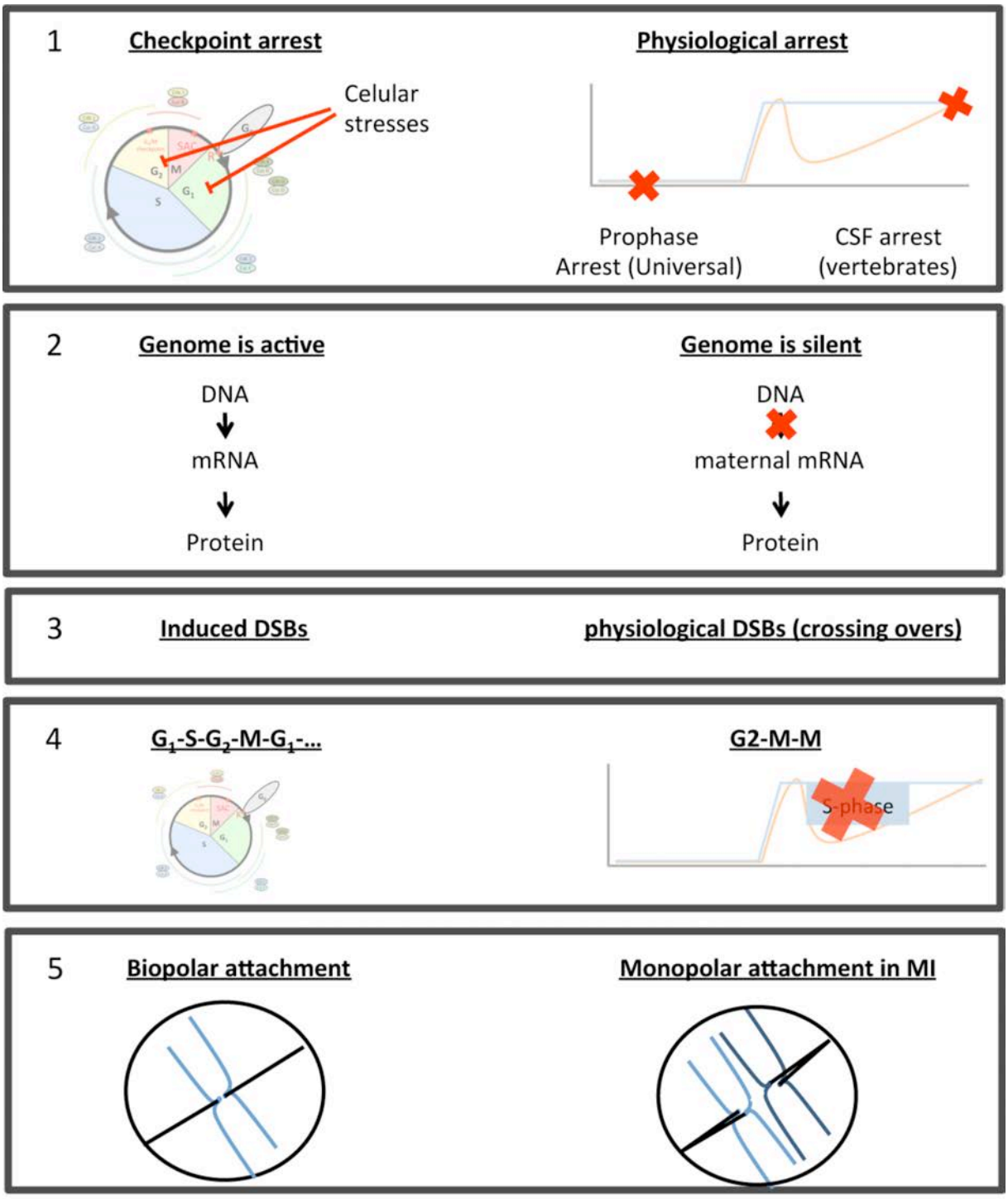

Fig. 13: Differences between mitosis and meiosis

In this figure are listed the main differences between the mitotic cell cycle and the meiotic division.

to species where fertilization occurs at prophase I (some sponges, some nematodes, some crustacean) (**Fig. 13**).

ii. Silencing of the genome

The transcriptional activities of the oocyte genome are strictly controlled during oogenesis. When diploid germ cells, oogonia, enter prophase I of meiosis, the chromosomes get condensed, impairing any transcriptional activity during the prophase period from leptotene to pachytene. The oocyte then arrests at the diplotene stage of prophase I and some specific portions of the chromosomes undergo decondensation, leading to the formation of uncondensed loops of chromatin, hence accounting for the name of "lampbrush" chromosomes (Hill *et al.* 1980). During the first part of the oocyte growth period, the loops of the lampbrush chromosomes are transcribed at very high rates and their products are accumulated in the oocyte as stores for the future embryo (Hill *et al.* 1980). The transcription is then shut off around the stages IV-V of oogenesis in *Xenopus*. The loops disappear by chromatin condensation and the transcription becomes undetectable. The full-grown oocyte ready for the prophase release and the ovulation is therefore transcriptionally silent (**Fig. 13**).

While the mitotic cell cycle is tightly controlled by genome expression (transcription and translation), the incubation of amphibian oocytes with α-amanitin, an inhibitor of RNA Pol II and III, has no effect on meiosis resumption (Wasserman *et al.* 1974). Identical results were obtained with the replication inhibitor, aphidicolin. *Xenopus* oocytes can be manually enucleated by just pricking the oocyte in a hyperosmotic external medium. Under these conditions, the germinal vesicle is extruded and the oocyte heals quickly. When enucleated oocytes are stimulated by the external stimuli inducing the prophase release, they support the activation of MPF, showing that the transduction pathway leading to meiosis resumption is independent of transcription, and also of every nuclear component (Masui *et al.* 1971). These results were not surprising, given the fact that the transcription is silent in these cells.

On the other hand, the oocyte supports an active global protein synthesis activity, translating the so-called "maternal" mRNA stored in the cytoplasm, which is stimulated during the meiotic divisions. The inhibition of protein translation with either Cycloheximide (CHX) or emetine totally abolishes meiosis resumption in response to progesterone in *Xenopus* (Wasserman *et al.* 1975). However, the dependency of the completion of meiotic divisions towards protein synthesis depends on species (See chapter: I-B.d.iii.2. "The protein synthesis"). In all species studied so far (invertebrates and vertebrates), one mechanism involved in the control of protein synthesis in the oocyte is the mRNAs polyadenylation. This process is mediated through a 3'UTR sequence called cytoplasmic polyadenylation elements (CPE), which can be recognized by CPE binding proteins (CPEB). Several

molecular pathways can modulate CPEB activation and consequentially the translation of mRNAs. In addition, pathways such as the MAPK pathway or PKA activity can modulate the meiotic divisions, but through non-genomic effects. In conclusion, the meiotic divisions are independent of transcription. They rely on post-translational modifications and, in some species, on protein translation (**Fig. 13**).

iii. Recombination and post-recombination events

The female diploid germ cells enter into meiosis and arrest in prophase I. Prophase I is highly specific of meiosis and is classically divided into several stages: leptotene (chromosome condensation), zygotene (pairing of homologous chromosomes), pachytene (chromosomal crossover), and diplotene (division arrest). The meiotic recombination (crossing-over) exchanges the genetic materials between two homologous chromosomes. The crossing-over, occurring during the pachytene stage of prophase I, allows the independent segregation of loci present on the same chromosome, promoting the formation of chromosomes composed by new combination of alleles. Importantly, recombination also produces the formation of chiasma that are necessary for holding homologous chromosomes together. This physical attachment persists until metaphase I and is fundamental for chromosome segregation during meiosis I. The molecular recombination machinery itself is largely shared by both the meiotic recombination and the homologous recombination repair during the mitotic cell cycle (Bzymek *et al.* 2010). However, in the case of meiosis, double-strand breaks (DSBs) are produced by a physiological cellular process, rather than resulting from accidental damages as in the mitotic cell cycle (**Fig. 13**). Furthermore, the sites of DSBs at meiotic recombination are not random, but often clustered at hot spots (Lichten *et al.* 1995). Of course, the recombination partners are homologous chromosomes in meiosis whereas recombination occurs between sister chromatids of a single chromosome during the mitotic cycle.

The post-recombination events are also very different between mitosis and meiosis. In mitosis the chiasma are resolved quickly. In contrast, in meiosis, they last until metaphase I that takes place some weeks, or months or even years (human) later, depending on the length of the prophase arrest that varies according to species. This prolonged arrest is linked to the so-called maternal age effect in humans (Hassold *et al.* 2009), which corresponds to an increase in aneuploidy incidence as the age of the mother increases. In mitosis, the cohesion between sister chromatids is established in S-phase and stays on chromosomes until mitosis (Uhlmann *et al.* 1998). During meiosis, the gradual loss of cohesins between sister chromatids and homologous chromosomes during the prolonged prophase arrest increases the frequency of missegregation. This “cohesion fatigue” could be the cause of the increased aneuploidy incidence correlated to the age of the mother.

iv. The succession of two M-phases with no intervening S-phase

During the mitotic cell cycle, chromosomes replication and segregation alternate maintaining the stability of the ploidy. In contrast, female meiosis consists in two cellular asymmetric divisions with no intervening S-phase, reducing the ploidy of the cell by half. The suppression of the S-phase is achieved by a special regulation of Cdk1 activity during the MI-MII transition, which is partially due to a specific germ line kinase pathway: the Mos/MAPK pathway. As this issue is directly addressed by my thesis project, it is described in more details in (Chapter I.B.e.iii. "The molecular control of DNA replication") (**Fig. 13**).

v. The separation of homologous chromosomes

A major specific point of meiosis is the segregation of the homologous chromosomes during meiosis I in contrast to the segregation of sister chromatids that occurs during mitosis. The meiosis I is therefore reductional as it divides by half the cellular ploidy. The Meiosis II is equational and as in mitosis, segregates sister chromatids in two daughter cells. The ability to segregate homologous chromosomes relies on three mechanisms. The first one is the position of chromosomes on the meiotic metaphase plate. Homologous chromosomes being paired together, they face each other on the plate, instead to be aligned next to each other as in mitosis. The second one is the organization of kinetochores. While sister kinetochores of one chromosome attach to the opposite poles in mitosis, sister kinetochores of each homologous chromosome attach to one pole during metaphase I (**Fig. 13**). The third difference is the stepwise removal of cohesins from chromosomes. During the mitotic cell cycle, cohesins connect sister chromatids together until the metaphase to anaphase transition, when they are removed by phosphorylation and cleavage. In contrast, in meiosis I, homologous chromosomes are connected both by chiasma and cohesins, while the sister chromatids of each chromosomes are linked by centromeric cohesins. The issue of meiosis I is to separate homologous chromosomes, but to preserve the attachment of sister chromatids of each of them. At the onset of anaphase I, chiasma are resolved and the distal cohesins, which connect together the homologous chromosomes, are phosphorylated, and then degraded. However, the centromeric cohesins are protected from degradation, avoiding sister chromatids separation. This protecting mechanism depends on the recruitment of Shugoshin (Sgo) and the PP2A phosphatase to centromeres (Kitajima *et al.* 2004, Kitajima *et al.* 2006, Riedel *et al.* 2006). Sgo/PP2A protects centromeric cohesins by maintaining them under a dephosphorylated state.

Another key difference regards the spindle formation. Remarkably, meiotic spindles form without centrosomes in the oocytes of most of animals, including human, mouse, *Xenopus*, *Drosophila* and *C.*

elegans, conferring the typical barrel-shaped morphology of oocyte meiotic spindles. This is specific of oocytes, since spermatocytes still contain centrosomes that participate to spindle formation. How centrosomes are eliminated during the early steps of the prophase oocyte growth is poorly documented. Recent evidence suggests the existence of oocyte-specific mechanisms compensating for a lack of centrosomes (Goshima *et al.* 2008).

A last meiotic specificity is the involvement of the SAC in the regulation of meiotic division that depends on the species. A functional SAC is clearly operating in mouse oocytes undergoing meiosis, since the complete disruption of meiosis I spindle using nocodazole, causes metaphase I arrest with intact chromosome bivalents (Wassarman *et al.* 1976a, Wassarman *et al.* 1976b, Schultz *et al.* 1977, Eichenlaub-Ritter *et al.* 1989, Soewarto *et al.* 1995, Brunet *et al.* 1999, Homer *et al.* 2005). Upon nocodazole removal, the oocyte rebuilds a metaphase spindle and proceeds to anaphase and cytokinesis by expelling the 1st polar body. Moreover, deficiency in SAC proteins increases mouse egg aneuploidies both *in vivo* and *in vitro* (Homer *et al.* 2005, Leland *et al.* 2009, Li *et al.* 2009, McGuinness *et al.* 2009, Hached *et al.* 2011). In contrast to mouse oocytes, the disruption of microtubules with nocodazole does not cause any metaphase I arrest in *Xenopus* oocytes (Shao *et al.* 2013), suggesting that SAC does not monitor the MI spindle of *Xenopus* oocytes.

b. Oogenesis and meiotic maturation

In females, the meiotic maturation is the last step of oogenesis. This process corresponds to the differentiation of a diploid germ cell progenitor in a mature fertilizable oocyte. During the embryonic development, the primordial germ cells migrate to the embryonic gonads. These germ cells proliferate by mitosis inside the gonad. After a last pre-meiotic-S-phase, they enter into meiosis and oocytes then become arrested at the diplotene stage of prophase of the first meiotic division in all animal species. The mitotic proliferation period preceding entry into meiosis I is completed before birth in mammals. Therefore the gonads of new-born mammal females contain a fixed and limited amount of oocytes with no capacity to self-regenerate. In contrast, proliferation of germ cells occurs all over the life-span of most of the animals, except mammals.

i. The oocyte growth period

Depending on the species, the prophase I arrest lasts from months to years. During this long period, the oocyte growths by accumulating all the mRNA and proteins required for the resumption of meiosis and for the development of the future embryo. The growth is particularly spectacular in oviparous species where the embryonic development entirely relies on maternal nutrient storage

until the new organism is able to feed by itself. The oocyte growth in oviparous species does not only depend on the intrinsic oocyte anabolic activities but also on the uptake of proteins, called vitellogenesis, supplied by the female liver through the blood flow. In *Xenopus*, the oocyte growth period (lasting 2-3 years) has been divided in a first period relying on intrinsic oocyte synthesis, called pre-vitellogenesis, followed by a period of intense growth due to the vitellogenin uptake and called vitellogenesis. Six stages have been described according to the diameter of the oocyte as well as the external appearance of the growing oocyte (Dumont 1972). During pre-vitellogenesis, oocytes are firstly colourless (Stage I) then become white and opaque (Stage II). Stage III corresponds to the beginning of vitellogenesis with active uptake of the yolk and to the initiation of pigment synthesis. The oocyte is almost fully dark. At the Stage IV, the animal (dark) and vegetal (white) hemispheres are individualized due to a targeted localization of cellular pigmentation. As the oocyte reaches the Stage V, the accumulation of the yolk starts to decrease and the cell almost reaches its maximal size. The Stage VI, referred as post-vitellogenic stage, is characterized by the presence of a white equatorial band between the two hemispheres. The post-vitellogenic oocyte is the biggest cell of the whole organism, with a diameter comprised between 1200 and 1300 µm. At the end of vitellogenesis, only the stage VI oocytes can resume meiosis and proceed through meiotic maturation.

ii. Meiotic maturation

The meiotic maturation corresponds to the prophase release, which is initiated concomitantly with ovulation. In Vertebrates, ovulation is launched by the Luteinizing Hormone (LH), and promotes the release of the oocyte from its surrounding follicle cells and from the ovaries. In *Xenopus*, LH also induces the synthesis of a steroid hormone, progesterone (Pg), by the follicle cells. Pg is released from the follicle cells and acts on the oocyte both in a paracrine manner and through gap junctions connecting the follicle cells and the oocyte. In Fish and amphibian, this steroid promotes the meiosis resumption of the oocyte.

1. Morphological events

In *Xenopus* oocytes, the first meiotic division starts after a latent period ranging from 2 to 6 hours after Pg stimulation with the nuclear envelope breakdown, also called GVBD for Germinal Vesicle Break Down, which is visualized by the appearance of a white spot at the animal pole of the cell. Soon after, a giant microtubular array (100 µm) organizes at the base of the disrupting nucleus and gathers the condensed bivalent chromosomes. This microtubular array migrates toward the animal pole where it is reorganized into a short bipolar metaphase I spindle (10 µm), oriented parallel to the

oocyte surface. The spindle then rotates and the first polar body is extruded 90 minutes following GVBD, marking the completion of meiosis I and reducing by half the cellular ploidy. Thereafter, oocytes proceed immediately through the second meiotic division by organizing a metaphase II spindle oriented parallel to the oocyte surface in the absence of nuclear envelope reformation. The metaphase II spindle then rotates and become orthogonal to the oocyte cortex where it anchors 3 hours after GVBD initiation (Gard 1992). The oocyte arrests at that stage for several hours, until fertilization. In the absence of fertilization, it dies by apoptosis (Du Pasquier *et al.* 2011).

2. Profile of Cdk1 activity

The succession of the two meiotic divisions depends on two successive waves of Cdk1 activation (**Fig. 14**). The first activation of Cdk1 promotes GVBD. After the metaphase I, MPF is inactivated due to the ubiquitin-dependent degradation of Cyclin B, allowing anaphase I and the exit from meiosis I. However, Cdk1 is not fully inactivated due to a concomitant degradation and synthesis of Cyclin B. This residual level of active Cdk1 prevents the decondensation of DNA and the reformation of nucleus. Cdk1 activity quickly raises again due to the arrest of Cyclin degradation and the synthesis of new Cyclins B that induce entry into meiosis II signalled by the formation of a metaphase II spindle, without prophase II. In vertebrates, the activity of MPF is then stabilized at a high level by a cytostatic factor called CSF that ensures the arrest at metaphase II (**Fig. 14**).

c. The arrest in prophase

In Vertebrates, the arrest at prophase I is controlled by a high intracellular concentration of cyclic AMP (cAMP), which stimulates the activity of the cAMP-dependent protein kinase A, PKA. This regulation of the meiotic progression is reminiscent of the control exerted by cAMP during the G_2/M transition of some cell lines. In various cell lines, cAMP levels fluctuate during the cell cycle, being high in interphase and low in mitosis (Burger *et al.* 1972, Fernandez *et al.* 1995). Moreover, the drop in cAMP levels or the inhibition of PKA activity occurring in late G_2 are both required for the entry in M-phase (Stambrook *et al.* 1976, Lamb *et al.* 1991, Kurokawa *et al.* 1998). While it is well established that the cAMP-PKA pathway keeps Cdk1 inactive in prophase oocytes, the molecular connection between PKA and MPF in oocytes as well as in somatic cells remains poorly documented.

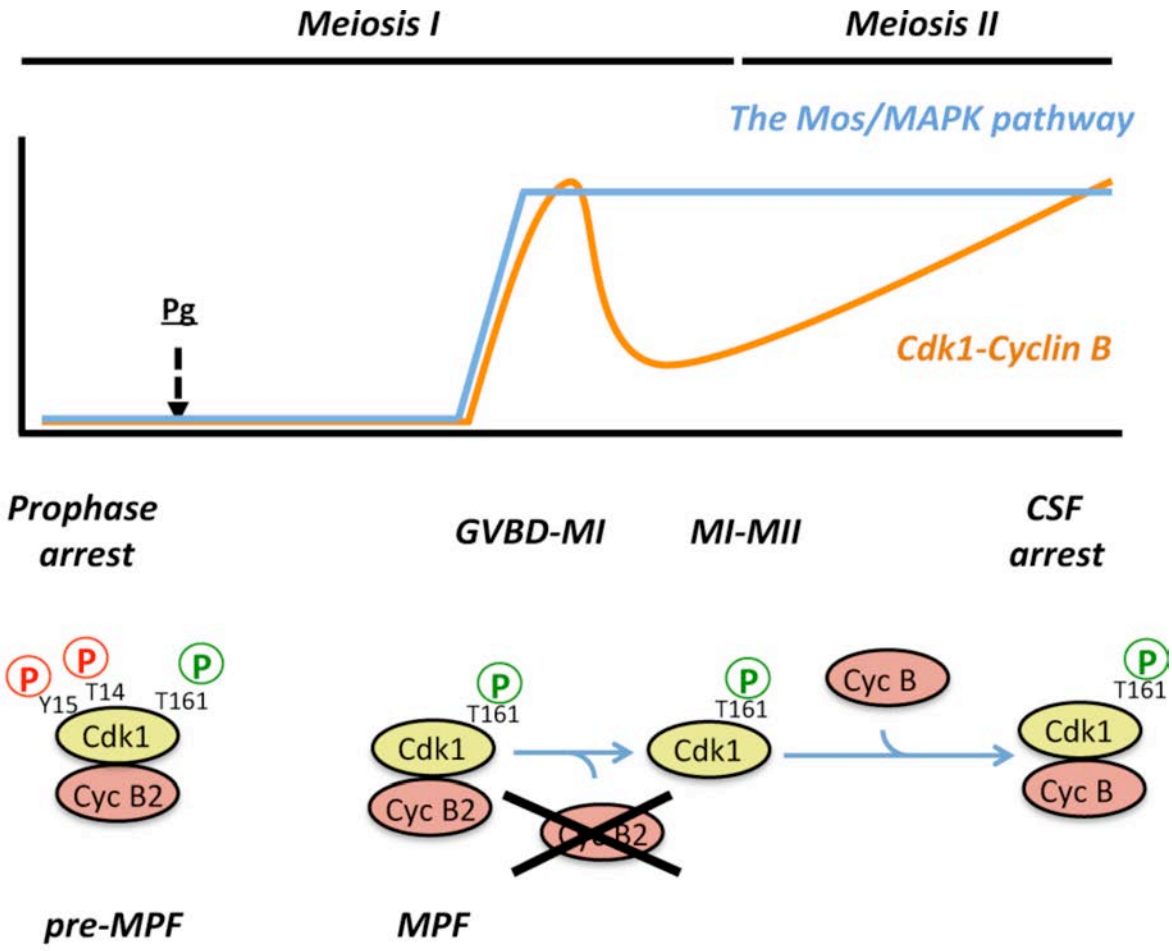

Fig. 14: Profile of Cdk1 activation during meiotic maturation

The succession of the two meiotic divisions depends on two consecutive waves of Cdk1 activation. The first activation of Cdk1 promotes GVBD. After the metaphase I, MPF is inactivated due to the ubiquitin-dependent degradation of Cyclin B, allowing anaphase I and the exit from Meiosis I. Cdk1 activity raises again due to the accumulation of newly synthetized Cyclin B, which induces the progression in Meiosis II. In vertebrates, the activity of MPF is then stabilized at a high level by a cytostatic factor called CSF.

A second essential kinase activity for meiotic maturation is the Mos/MAPK pathway. This pathway is activated at GVBD following the accumulation of the kinase Mos and remains active until fertilization. Atthat time, Mos is degraded and the Mos/MAPK pathway fully inactivated.

i. cAMP and PKA activity

In somatic cells, cAMP is a second messenger that regulates a plethora of cellular functions including gene expression and proliferation. cAMP is produced from ATP by the enzymes from the family of the adenylate cyclases (AC) and is degraded in 5'-AMP by the members of the phosphodiesterase family (PDE). The direct target of cAMP is the cAMP-dependent protein kinase A (PKA), an heterodimer composed of two types of subunits: two catalytic subunits (PKA-C) and two regulatory subunits (PKA-R). The PKA-C subunits contain the active catalytic site, an ATP-binding site and a binding domain for PKA-R subunits. The PKA-R subunits bind to each other in an anti-parallel orientation to form a homodimer. Each monomer possesses additional domains: one for the binding of cAMP, the second one for the interaction with a PKA-C subunit, the third one being an "auto-inhibitory" domain, which serves as a substrate or pseudosubstrate for PKA-C. When the intracellular concentration of cAMP is low, PKA-R dimer binds to PKA-C and PKA is inactive. When the level of cAMP increases, cAMP binds to PKA-R dimer and releases it from the PKA-C subunits, leading to the activation of PKA.

In mammal prophase-arrested oocytes, the source of cAMP results from the influx of cAMP through gap junctions connecting follicular cells to the oocyte, but also from the endogenous production of cAMP within the oocyte induced by the adenylate cyclase activation through G-protein coupled receptor, GPCR3 (Reviewed in Mehlmann 2005). Moreover, in rodent oocytes, the influx of cGMP from follicular cells to the oocyte prevents the activation of the PDE3, ensuring a high cAMP level (Norris *et al.* 2009). In *Xenopus* oocyte, the adenylate cyclase activity maintains high levels of cAMP (Mulner *et al.* 1979b, Finidori-lepicard *et al.* 1981, Sadler *et al.* 1981a) that in turn activate PKA (Maller *et al.* 1977, Wang *et al.* 2004). Any treatment that increases either the intracellular cAMP levels or the activity of PKA totally abolishes Cdk1 activation upon hormonal stimulation (Ozon *et al.* 1978, Huchon *et al.* 1979, Ozon *et al.* 1979a, Ozon *et al.* 1979b). Reversely, inhibiting the PKA activity is sufficient for triggering meiosis resumption independently of the hormonal stimulation (Maller *et al.* 1977) (Huchon *et al.* 1981c, Rime *et al.* 1992b, Daar *et al.* 1993) (**Fig. 16**). The cAMP-PKA pathway is already functional in stage IV oocytes since progesterone, although not able to promote Cdk1 activation and GVBD, leads to the inhibition of adenylate cyclase, either measured *in vitro* with purified adenylate cyclase from stage IV oocytes or assayed *in vivo* by a drop in cAMP level (Mulner *et al.* 1983, Sadler *et al.* 1983a). Thus, cAMP and PKA are key actors for the oocyte arrest at prophase by keeping Cdk1 inactive. Importantly, the effects exerted by PKA on meiotic division progression are independent of transcription on the contrary to somatic cells.

ii. Statute of Cdk1 and its direct regulators

In prophase oocytes, Cdk1 is kept inactive by two mechanisms depending on the species: either the absence of its activatory partner, the Cyclin B, and or its inhibitory phosphorylations at T14 and Y15. In some fishes, like Goldfish, Cyclin B is not expressed in prophase. The activation of Cdk1 then relies on Cyclin B synthesis that associates with monomeric Cdk1 that becomes phosphorylated at T161 upon Cyclin binding (Tanaka *et al.* 1995, Yamashita *et al.* 1995). In mouse, the activation of Cdk1 is initiated through the dephosphorylation at Y15 of the stockpile of pre-MPF. This activity level promotes GVBD but is not high enough to sustain the formation of the metaphase I spindle. Cyclin B must be synthesized to elevate the level of Cdk1 activity and to proceed through the meiosis I (Hampl *et al.* 1995b, Winston 1997, Polanski *et al.* 1998). In *Xenopus*, both mechanisms are functional as suggested by the presence of two different inactive pools of Cdk1: a monomeric form of Cdk1, which is ready to be activated because a part of it is already phosphorylated at T161, and a dimeric form of Cdk1 associated with the Cyclin B that forms the pre-MPF (Kobayashi *et al.* 1991, Hochegger *et al.* 2001, De Smedt *et al.* 2002) (**Fig. 15**). In pre-MPF complexes, Cdk1 is phosphorylated at T161, T14 and Y15, these two last phosphorylations maintaining it in an inactive state (Gautier *et al.* 1989, Mueller *et al.* 1995b, De Smedt *et al.* 2002). The pre-MPF is constantly formed in prophase due to a slow synthesis of Cyclin B that associates with monomeric Cdk1 present in large excess, and are immediately phosphorylated by Myt1 (Rime *et al.* 1995, Gaffre *et al.* 2011).

1. Wee1 and Myt1

In *Xenopus* prophase-arrested oocytes, Wee1 is expressed from stage I to stage IV during the growth period and is responsible for the inactivation of Cdk1 inside pre-MPF complexes that accumulate during this period (Nakajo *et al.* 2000). From stage IV to VI, Wee1 is no longer expressed at the protein level and it reappears during late meiotic maturation, when the oocyte enters in the second meiotic division (Murakami *et al.* 1998, Charlesworth *et al.* 2000, Nakajo *et al.* 2000). In contrast, the kinase Myt1 is constantly expressed starting from stage IV, where its activity takes over the one of Wee1, and during the whole meiotic maturation. In prophase oocytes, Myt1 is hypophosphorylated and active (Palmer *et al.* 1998, Gaffre *et al.* 2011). In stage VI resting oocytes, the slow rate of Cyclins B2 and B5 synthesis induces the formation of Cdk1-Cyclin B complexes that are inactivated by Y15 phosphorylation (Rime *et al.* 1995)(Gaffre *et al.* 2011). Myt1 inhibition with either PD0166285 or blocking antibodies induces meiosis resumption in the absence of progesterone (Ruiz *et al.* 2008, Gaffre *et al.* 2011). Conversely, the overexpression of Myt1 delays Cdk1 activation in response to progesterone in a dose-dependent manner (Gaffre *et al.* 2011). Therefore, the activity of Myt1 is

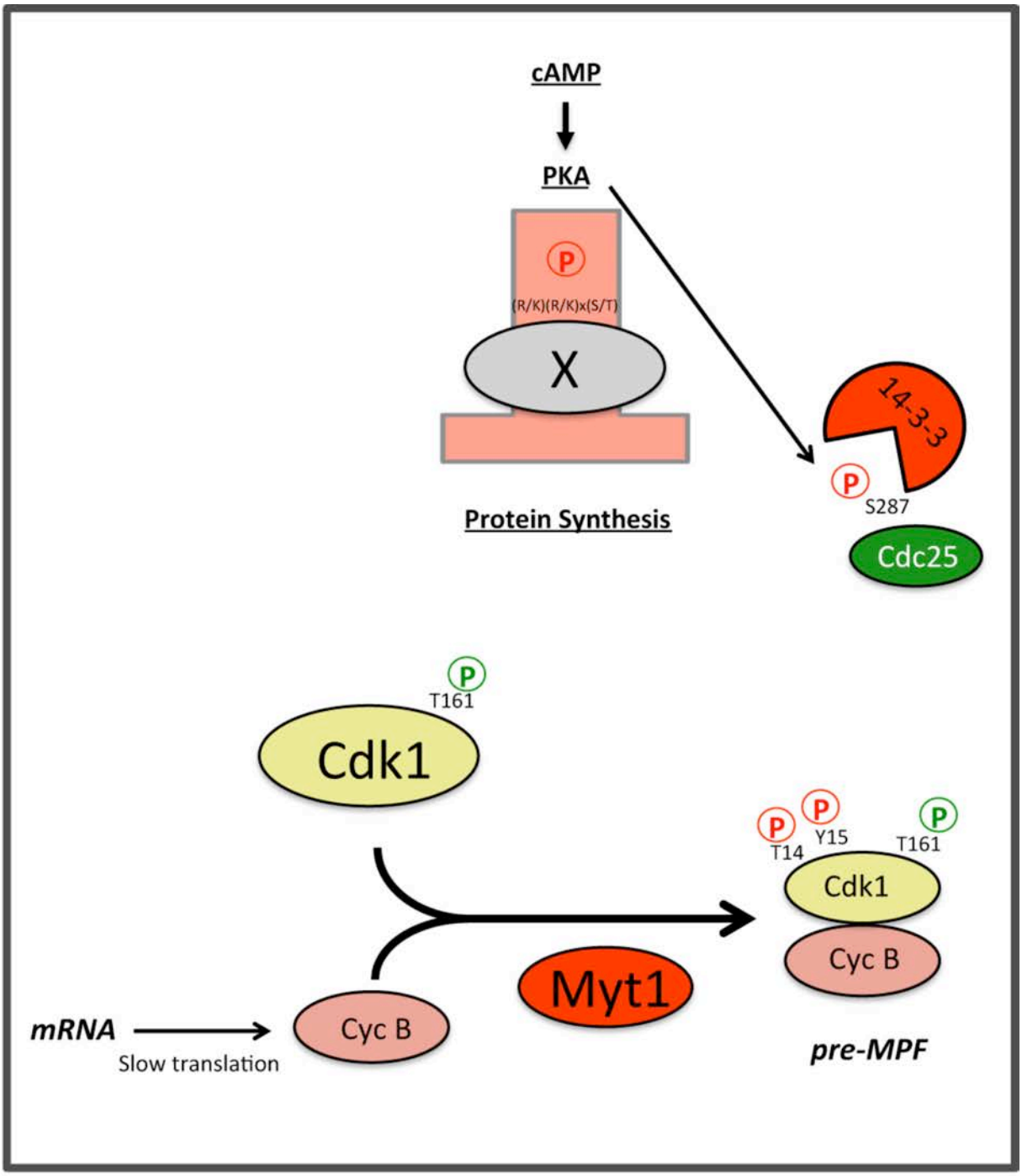

Fig. 15: The molecular control of the arrest in prophase

Xenopus prophase-arrested oocytes contain two pools of inactive Cdk1: a large excess of free Cdk1 and a minority already bound to Cyclin B (pre-MPF). The pre-MPF is continuously formed during the prophase arrest because of the slow synthesis of new Cyclins. The activity of pre-MPF is inhibited by the kinase Myt1 that is active and phosphorylates Cdk1 at T14 and Y15 and because Cdc25 is inactive. The arrest in prophase is maintained by a high intracelulular concentration of cAMP and high PKA activity. The molecular pathway that connects PKA activity to MPF inhibition has not been completely elucidated.

required in prophase to inhibit by phosphorylation the newly Cdk1-Cyclin B complexes that are permanently formed during this long-lasting arrest (**Fig. 15**).

On the contrary to *Xenopus* oocytes, the kinase Wee1 is present in mice prophase-arrested oocytes and its activity is enhanced following its phosphorylation by PKA at S16 (Han *et al.* 2005). Whether PKA can regulate Myt1 activity during prophase arrest in *Xenopus* oocytes is unknown and deserves to be investigated.

2. Cdc25

Two genes encoding the phosphatase Cdc25 have been identified from a *Xenopus* oocytes cDNA library (Izumi *et al.* 1992, Kumagai *et al.* 1992, Okazaki *et al.* 1996). One of them is clearly homologous to human Cdc25A while the other one is closer from human Cdc25B. This latter form is thought to be the physiological regulator of Cdk1 during oocyte meiotic maturation and despite its closer similarities with human Cdc25B, has been called Cdc25C in *Xenopus* (Izumi *et al.* 1992, Kumagai *et al.* 1992, Okazaki *et al.* 1996). In *Xenopus* oocyte, Cdc25C is expressed at the protein level under an inactive form during the whole growth period (Dunphy *et al.* 1991, Gautier *et al.* 1991, Jessus *et al.* 1992, Nakajo *et al.* 1999, Nakajo *et al.* 2000, Oe *et al.* 2001). As in somatic cells, the mechanism responsible for keeping Cdc25C inactive in prophase depends on its phosphorylation at S287 (Duckworth *et al.* 2002) (**Fig. 15**). While Chk1 and Chk2 were at first proposed as the kinases responsible for the S287 phosphorylation of Cdc25C, as in mitotic cells (Nakajo *et al.* 1999, Oe *et al.* 2001), it was then proposed that this phosphorylation is under the control of PKA (Duckworth *et al.* 2002). The injection of PKI promotes the dephosphorylation at S287 of Cdc25C and leads to its dissociation from 14-3-3 proteins (Duckworth *et al.* 2002). Cdc25C dephosphorylation was further observed in response to progesterone and was impeded following the incubation of oocytes in the presence of IBMX, a phosphodiesterase inhibitor (Duckworth *et al.* 2002). Furthermore, overexpressing PKA-C totally impairs the ability of overexpressed Cdc25C in promoting meiosis resumption whereas the expression of a non phosphorylatable form of Cdc25C, the S287A-Cdc25 mutant, is able to bypass the lock exerted by PKA. Altogether, these results strongly suggest that Cdc25C is a physiological substrate of PKA in prophase oocytes and that the PKA-dependent phosphorylation at S287 prevents its activation during the prophase arrest (**Fig. 15**). In agreement with these results, *Xenopus* WT-Cdc25C is a weak inducer of meiosis resumption compared to S287A-Cdc25 mutant (Nakajo *et al.* 1999, Duckworth *et al.* 2002). In contrast, overexpressing human or *Xenopus* Cdc25A activates Cdk1 independently of progesterone stimulation (Rime *et al.* 1994, Okazaki *et al.* 1996, Duckworth *et al.* 2002). The difference in the ability to promote meiosis resumption between these two isoforms of Cdc25, A and C, can be explained by the absence of the

S287 within Cdc25A, which therefore cannot be phosphorylated and inhibited by PKA in prophase (Okazaki *et al.* 1996). The negative effect of S287 phosphorylation on Cdc25C activity is attributed to its interaction with 14.3.3 proteins and its nuclear import inhibition. However the precise role of this regulation has to be further investigated in the oocyte context where the regulation of nuclear localization of the main actors does not appear to be instrumental for controlling meiotic divisions. Especially, the activation of Cdk1 does not require the nucleus, as it occurs in response to Pg in enucleated *Xenopus* oocytes. Opposite to S287, the phosphorylation of many other S and T residues inside Cdc25C is required to activate its phosphatase activity. Those residues are not phosphorylated in prophase oocytes, leading to an inactive hypo-phosphorylated form of Cdc25.

In summary, the arrest at prophase I is regulated by a high level of PKA activity that indirectly keeps Cdk1 inactive. Pre-MPF molecules are kept inactive by phosphorylation at T14 and Y15 by high Myt1 activity which overrides Cdc25 phosphatase activity (**Fig. 15**).

d. Mechanisms controlling the release from the prophase arrest

i. The competence to resume meiosis

The competence to resume meiosis is acquired during the very last steps of vitellogenesis. Stage IV oocytes are unable to mature in response to progesterone despite progesterone ability to downregulate cAMP-PKA signalling pathway (Mulner *et al.* 1983, Sadler *et al.* 1983a). Furthermore, the injection of Cyclin B2, Cdc25 or Okadaic acid, at a concentration sufficient to trigger meiotic maturation in stage VI oocytes, are all unable to promote Cdk1 activation in stage IV oocytes (Reynhout *et al.* 1975, Sadler *et al.* 1983a, Rime *et al.* 1995). However, injecting active MPF by cytoplasm transfer in stage IV oocytes induces GVBD, but with only a low Cdk1 activity, which is not able to promote the formation of a metaphase I spindle (Rime *et al.* 1991). At GVBD inactive pre-MPF not dephosphorylated at Y15 is still present, suggesting that only the transferred molecules have been activeted but did not activate the endogenous pre-MPF stockpile of the oocyte (Rime *et al.* 1991). Therefore, in stage IV oocytes, the cAMP-PKA pathway downregulation is not connected to the activation of MPF and the inability of stage IV oocytes to resume meiosis is due to a defect within the MPF autoamplification, a preocess necessary to dephosphorylate completely Cdk1 at T14 and Y15. Plx1 is not expressed in stage IV oocytes and its ectopic expression was shown to restore the MPF autoamplification loop in these small oocytes as long as PP2A is inhibited by OA injection (Karaiskou *et al.* 2004). This suggests that the pathway induced by Pg and inhibiting PP2A phosphatase, a requirement for MPF autoamplification, is not active in these small growing oocytes.

The incompetency to resume meiosis is therefore due to a defect in the pathway leading to Cdk1 full activation as well as the inability to regulate PP2A, both of them being possibly connected.

ii. Hormones and Receptors

The release of the oocyte into the oviduct (or ovulation) is promoted by the pituitary gonadotropin LH (luteinizing Hormone) that concomitantly induces the secretion of steroid hormones responsible for meiosis resumption from the follicular cells surrounding the oocyte. In *Xenopus* oocytes, progesterone is the physiological mediator of meiotic maturation (Haccard *et al.* 2012) but many different C_{19} and C_{21} steroids are able to induce meiotic maturation *in vitro* on defolliculated oocytes, such as testosterone, pregnenolone and many others, whatever they are physiological hormones or metabolic intermediates or antagonists of natural hormones or synthetic compounds (Baulieu *et al.* 1978).

The canonical way by which progesterone acts on cells, depends on Progesterone Receptors (PR) that are found in the cytoplasm and the nucleus of target cells. Classically, unliganded PRs reside mainly in the cytoplasmic compartment as inactive proteins. Upon ligand binding, PR are phosphorylated, dimerize and translocate to the nucleus where they act as specific transcription factors. They bind their target genes at specific palindromic progesterone-response elements (PREs) located in active chromatin (reviewed in Abdel-Hafiz *et al.* 2014). Then PRs activate or repress transcription depending on the promoter being regulated and on the cofactors that are recruited to this promoter. This classical model has been recently come into question with data showing that PR can be localized into the nucleus independently of the hormone, that the dimerization is not required for the PR-mediated transcription and that PR can act through cell membranes and cytoplasmic signalling pathways, independently of transcription (Boonyaratanakornkit *et al.* 2008, Jacobsen *et al.* 2012). Beside the binding to the ligand, PRs are also controlled by post-translational modifications including sumoylation, phosphorylation, ubiquitination and acetylation that modify the stability, the hormone sensitivity and the nuclear localization of both ligand and unliganded receptors (reviewed in Abdel-Hafiz *et al.* 2014). These modifications are initiated by signalling pathways activated at the level of cell membranes and, in turn, PRs modify the cell signalling through non-genomic mechanisms. These non-genomic effects are mainly driven by a small fraction of classical cytoplasmic liganded PRs associated with tyrosine kinases and other modules, but also by cell surface membrane PRs (mPR) (reviewed in Abdel-Hafiz *et al.* 2014). The mPRs posses seven transmembrane domains and some conserved cysteines for disulfide bounding (Zhu *et al.* 2003, Thomas *et al.* 2004). These receptors bind progesterone outside of the cell and activate a G-protein coupled pathways inside the cell (Zhu *et al.* 2003, Thomas *et al.* 2007).

In *Xenopus* oocytes, the molecular identity of the PR remains a mystery since half a century. This receptor has particular features compared to classical PRs. The fact that multiple different steroids induce meiosis resumption suggests either the presence of original steroids receptors with a very low specificity or the presence of more than one receptor. Moreover, progesterone acts at µM concentration (the concentration effectively measured in the *Xenopus* ovary, (Haccard *et al.* 2012)), as well as the other steroids, meaning more than 10^3 order of magnitude higher than the active concentrations of these hormones in the blood. This implies the presence of an original low affinity receptor. As already mentioned, the mechanism induced by progesterone is independent of transcription, meaning that, even so the classical PR would be involved, it would act with different targets than in somatic cells. Moreover, the mechanism induced by progesterone in *Xenopus* oocytes relies on membranes (Maller 2001). While the injection of a lipidic solution containing progesterone induces meiosis resumption (Tso *et al.* 1982), injecting progesterone in an aqueous solution does not trigger meiosis resumption (Jacobelli *et al.* 1974). Moreover, when progesterone is coupled to sepharose beads or to high molecular weight polymers that cannot enter within the cell, progesterone is able to promote meiosis resumption (Ishikawa *et al.* 1977, Godeau *et al.* 1978). Altogether, these results suggest that progesterone and other steroids have to reach a target that is present either in the plasma membrane or in intracellular membranes connected to the plasma membrane.

Several mRNAs encoding various steroid receptors are stored in oocytes. Among them, two canonical nuclear progesterone receptors (nPR), xPR-1 and xPR-2, have been cloned in addition to one mPR (Tian *et al.* 2000, Bagowski *et al.* 2001, Liu *et al.* 2005b). The knock-down of *Xenopus* xPR1 with antisense oligonucleotides strongly delays meiosis resumption in response to progesterone but does not abolish this process (Tian *et al.* 2000). This work has been a subject of controversy. There was no evidence showing that antisense oligonucleotides really deplete endogenous xPR protein from the oocyte. Very recently, it has been shown that the knock-out of the classical progesterone receptor in zebrafish impairs ovulation but does not affect oocyte meiosis resumption induced by the steroid (Zhu *et al.* 2015). Finally, since progesterone can trigger Cdk1 activation in enucleated oocytes, nPR is unlikely to be the receptor mediating the non-genomic effects of progesterone, unless it reaches original targets specific of the oocyte. All data actually converge to exclude a role of the classical nuclear progesterone receptors in triggering meiotic maturation.

mPR is a seven transmembrane receptor that was firstly identified in zebrafish and goldfish (Zhu *et al.* 2003, Tokumoto *et al.* 2006). *Xenopus* oocytes also express mPR and this receptor efficiently binds progesterone with the expected affinity for meiotic maturation (Josefsberg Ben-Yehoshua *et al.* 2007). These features render this receptor family member a very attractive candidate for mediating progesterone effects on oocyte meiotic maturation. However, mPR does not bind testosterone and

therefore does not fulfil the criteria of low specificity. A possibility is that mPR plays a physiological role in initiating the signalling pathway induced by progesterone but that other receptors with different specificities are also involved. Another provocative hypothesis is that the oocyte would not express any steroid receptors and that active steroids, corresponding generally to the more hydrophobic molecules among steroids, enter the plasma membrane, modify the lipidic layers and lead to the clustering of membrane proteins, such as G-proteins, inducing a signalling pathway through this local molecular re-organization of the membrane.

It has to noted that insulin and IGF-1 are able to trigger *in vitro* oocyte meiotic maturation (El-Etr *et al.* 1979, Maller *et al.* 1981). In this case, it is well established that both factors act through the classical receptor of IGF-1 that is expressed in the plasma membrane of the oocyte (Zhu *et al.* 1998). Surprisingly however, it is also clear that once the receptor of IGF-1 is activated, it reproduces the early events induced by progesterone, i.e. a drop in cAMP due to the inhibition of adenylate cyclase, and a decrease in PKA activity, and then all the subsequent events leading to MPF activation. Whether IGF-1 is physiologically involved in oocyte meiosis resumption is not known, although it is noteworthy that elevated levels of IGF-1 are present in the *Xenopus* ovary.

iii. The limiting steps for meiosis resumption

Two limiting steps are necessary for meiosis resumption in full-grown oocytes: the downregulation of PKA activity in Vertebrates and, depending on the species, the synthesis of new proteins from maternal mRNA (**Fig. 15**).

1. The decrease of cAMP levels and the downregulation of PKA

The first intracellular events occurring within 10 minutes upon progesterone stimulation in *Xenopus* oocytes are the inhibition of the adenylate cyclase activity, the decrease of about 20% of the cAMP intracellular concentration and the consequential downregulation of PKA activity (Mulner *et al.* 1979a, Sadler *et al.* 1981b, Sadler *et al.* 1983b, Wang *et al.* 2004) (**Fig. 16**). These events are both necessary and sufficient for Cdk1 activation. The incubation of oocytes with inhibitors of PDEs, such as IBMX (3-isobutyl-1-methylxanthine) or Cholera toxin, which maintain a high level of cAMP in oocytes, totally abolishes the ability of progesterone to trigger meiosis resumption (Huchon *et al.* 1979, Maller *et al.* 1980, Thibier *et al.* 1982) (**Fig. 16**). Moreover, the inhibition of PKA, by injecting either recombinant PKA-R or an inhibitor of PKA, PKI, induces Cdk1 activation independently of progesterone whereas the injection of PKA-C prevents progesterone-induced Cdk1 activation (Huchon *et al.* 1981c) (Maller *et al.* 1977) (**Fig. 16**). This implies that some substrates of PKA are

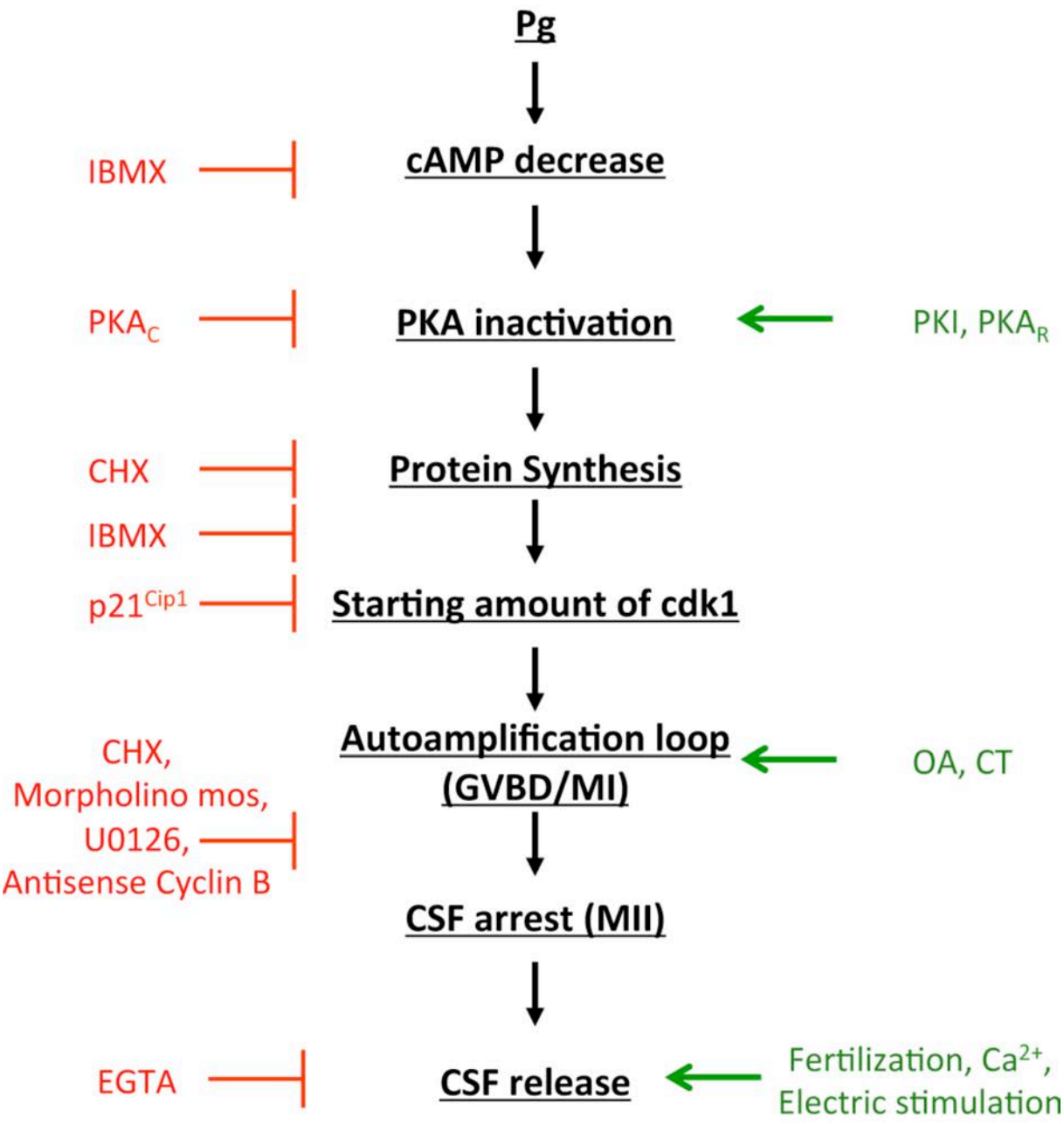

Fig. 16: The steps of meiotic division and the tools to activate/block them

Meiotic maturation steps are schematized in a linear signalling pathway. Inhibitors (in red) and activators (in green) are shown at the approximate place in which they are acting.

phosphorylated in prophase and must be dephosphorylated to promote Cdk1 activation. The identification of these PKA substrates in oocytes has been a challenge for many years. Cdc25C is clearly a PKA substrate in the oocyte (Duckworth *et al.* 2002). However, it does not correspond to the protein whose dephosphorylation is mandatory for Cdk1 activation. Indeed, the timing of Cdc25 dephosphorylation at the PKA site, S287, does not fit in the window of PKA inactivation that occurs within one hour following progesterone addition. Cdc25C is dephosphorylated at S287 at the time of MPF activation, just before GVBD, in a protein synthesis-dependent manner (Duckworth *et al.* 2002), strongly suggesting that this dephosphorylation takes place downstream of Cdk1 activation and is not a direct consequence of PKA inactivation. In the early 80s, biochemical attempts have led to the proposal that the PKA substrate in the *Xenopus* oocyte could be a 20 kDa acid- and thermo-resistant protein (Boyer *et al.* 1986) whose molecular identity had not been elucidated at that time. Therefore, the identification of the PKA substrate whose dephosphorylation is required for Cdk1 activation and meiosis resumption in Vertebrates remains an opened question.

2. The protein synthesis

In prophase-arrested oocytes, transcription is dormant and the synthesis of new proteins is only regulated at a translational level, from maternal mRNA. The requirement for protein synthesis during meiosis resumption varies according to species.

In species where prophase oocytes do not contain pre-MPF, such as some fishes, meiosis resumption relies entirely on Cyclins B synthesis (Katsu *et al.* 1993). In mouse, the activation of Cdc25 is independent of protein synthesis and sufficient for the dephosphorylation and the activation of pre-MPF leading to GVBD (Polanski *et al.* 1998, Ledan *et al.* 2001). However, the level of Cdk1 activity thus generated is not high enough to organize the first meiotic spindle and GVBD is immediately followed by a massive synthesis of Cyclin B, which binds monomeric Cdk1 and increases its activity (Hampl *et al.* 1995a, Winston 1997, Polanski *et al.* 1998, Hoffmann *et al.* 2006). In *Xenopus*, as in most mammals (except small rodents), although a high level of pre-MPF is stored in the cytoplasm and able to be activated by Cdk1 dephosphorylation at Y15 and T14, the synthesis of new proteins is necessary for Cdk1 activation. Oocytes stimulated by progesterone are unable to mature when protein synthesis is prevented by Cycloheximide (CHX) (Wasserman *et al.* 1975) (**Fig. 16**). In contrast, all components able to directly activate Cdk1 activity or the autoamplification loop, as Cyclins, Cdc25, or okadaic acid, act independently of protein synthesis (Rime *et al.* 1990, Gautier *et al.* 1991, Lee *et al.* 1992, Nebreda *et al.* 1995), confirming that protein synthesis is required prior Cdk1 activation. Interestingly, the inhibition of PKA induced by PKI injection is unable to activate Cdk1 in the presence of CHX (Huchon *et al.* 1981c). This suggests that the protein synthesis step is initiated downstream

PKA inhibition (**Fig. 15**). The molecular links between these two limiting steps in meiosis resumption have not yet been uncovered. However, three classes of proteins are known to be synthesized from maternal mRNA in response to progesterone and may be necessary for Cdk1 activation in oocytes: the Cyclins A and B, the kinase Mos and the protein Ringo/Speedy.

- ***The Cyclins***

– Cyclin A

In *Xenopus* oocytes, Cyclin A accumulates at a low level starting at GVBD (Kobayashi *et al.* 1991). While purified Cyclin A is able on its own to induce meiosis resumption by interacting with free Cdk1 (Swenson *et al.* 1986), the injection of antisense directed against Cyclin A1 mRNA did not block meiosis resumption in response to progesterone (Minshull *et al.* 1991). Hence, Cyclin A1 synthesis might not represent a requirement for MPF activation although it certainly participates to the activation of Cdk1. Its involvement in this process needs to be clarified.

– Cyclins B

In *Xenopus* oocytes, four subtypes of Cyclin B have been identified: B1, B2, B4 and B5 (Hochegger *et al.* 2001). Cyclins B2 and B5 are present in prophase-arrested oocytes and associate with monomeric Cdk1 to form the pre-MPF. Cyclins B1 and B4 are not expressed in prophase, or sometimes depending on the female individuals, at a low level. B1- and B4-Cyclins are synthesized in response to progesterone (Kobayashi *et al.* 1991, Hochegger *et al.* 2001), independently of Cdk1 activity because those proteins appear upon hormonal stimulation in prophase oocytes injected with $p21^{Cip1}$, a CDK inhibitor known to prevent Cdk1 activation and GVBD in *Xenopus* oocytes (Frank-Vaillant *et al.* 1999). Cyclin B1 also accumulates following PKI injection in $p21^{Cip1}$-injected oocytes, arguing that Cyclin B1 accumulation is under the control of PKA downregulation during meiosis resumption. Whether this accumulation results from an increase rate of synthesis or a stabilization of the protein remains unknown in *Xenopus* oocytes. Translation is clearly required as antisense oligonucleotides targeted against Cyclins B prevent Cyclin B1 accumulation. However in mouse oocyte, the inhibition of the APC^{Cdh1} is necessary for stabilizing Cyclin B1 and for meiosis resumption (Reis *et al.* 2006).

The potential importance of Cyclin B during meiosis resumption was initially revealed by injecting recombinant Cyclin B. When a sufficient amount of Cyclin B, able to overcome the activity of Myt1, is injected in prophase oocytes, Cdk1 is activated independently of progesterone and of protein synthesis (Nebreda *et al.* 1995). Exogenous Cyclin B binds to free T161-phosphorylated Cdk1 and triggers the full activation of Cdk1. Although, Cyclin B synthesis is sufficient for Cdk1 activation, this process is not however necessary for meiosis resumption since the injection of antisense

oligonucleotides directed against the four Cyclin B isoforms does not block meiosis resumption in response to progesterone (Hochegger *et al.* 2001, Haccard *et al.* 2006).

▪ *The kinase Mos*

The protein Mos is a serine/threonine kinase specifically expressed in female germ cells. Mos is responsible for the activation of MAPKK (MEK) that in turn activates MAPK then p90Rsk independently of PKA activity and protein synthesis (Nebreda *et al.* 1993, Posada *et al.* 1993, Shibuya *et al.* 1993, Dupre *et al.* 2011). This pathway is referred as the Mos/MAPK pathway. Mos mRNAs are present at a very low level in brain and testis and are very abundant in ovaries, particularly in oocytes. During oogenesis, Mos transcripts are already present at high concentration as early as in Stage I oocytes but the protein is never expressed during the growth period or in prophase-arrested full-grown oocytes (Sagata *et al.* 1988).

The translation of Mos mRNA is initiated in response to progesterone stimulation just before GVBD (Sagata *et al.* 1988). However, the accumulation of Mos, as well as the activation of MAPK, are only detected at GVBD when Cdk1 is activated. The involvement of the Mos/MAPK pathway in the activation of Cdk1 in *Xenopus* oocyte is supported by several lines of evidence. Firstly, the injection of recombinant Mos or its direct targets such as active thiophosphorylated MAPK or active $p90^{Rsk}$ induced GVBD and activates Cdk1 independently of progesterone stimulation (Yew *et al.* 1992, Haccard *et al.* 1995, Gross *et al.* 2001 and reviewed in Dupre *et al.* 2011). Secondly, the Mos-induced Cdk1 activation was shown to be independent of protein synthesis, provided that low concentrations of progesterone, unable to trigger meiosis resumption by itself, is added (Yew *et al.* 1992). Thirdly, inhibiting Mos synthesis with antisense oligonucleotides directed against its mRNA abolishes Cdk1 activation in response to progesterone (Sagata *et al.* 1988). Altogether, these results indicate that the Mos/MAPK pathway is necessary and sufficient for Cdk1 activation in response to progesterone.

These findings generated however controversy. In other species such as Mouse, Starfish and Goldfish, neither Mos synthesis nor MAPK activation are required for meiosis resumption (Hashimoto *et al.* 1994b, Verlhac *et al.* 1996a, Sadler *et al.* 1998, Kajiura-Kobayashi *et al.* 2000). Furthermore, Mos does not accumulate and MAPK is not activated when Cdk1 activation is prevented by injecting the CDK inhibitor, $p21^{Cip1}$ (Frank-Vaillant *et al.* 1999), strongly suggesting that the activation of the Mos/MAPK pathway relies on the activation of Cdk1. Finally, the inhibition of MAPK activation either by the pharmacological inhibitor of MEK activity, U0126, or by Geldanamycin, which acts on the chaperone protein HSP90, delays GVBD in response to progesterone and totally impedes Cdk1 activation following Mos injection in *Xenopus* oocytes (Fisher *et al.* 1999, Gross *et al.* 2000). Moreover, injecting specific morpholino antisense oligonucleotides directed against Mos mRNA

prevents both Mos synthesis and MAPK activation but does not block Cdk1 activation and GVBD, although both events are delayed (Dupre *et al.* 2002). Therefore, in *Xenopus* as in all species, the Mos/MAPK pathway is dispensable for meiosis resumption and the 1st acyivation of Cdk1.

- ***Ringo/Speedy***

The Speedy/Ringo protein was first identified in 1999 in two independent screens using a *Xenopus* oocyte cDNA library. The first one was aimed at identifying new mRNAs whose translation was able to stimulate meiosis resumption in this species. The second one was designed to find proteins conferring radio-resistance in yeast and thus promoting their release from G_2 when irradiated (Ferby *et al.* 1999a, Lenormand *et al.* 1999). Ringo is expressed in response to progesterone during meiotic maturation and must be downregulated after GVBD to ensure the proper progression of the oocyte in Meiosis II (Terret *et al.* 2001, Gutierrez *et al.* 2006). When overexpressed in prophase, Ringo/Speedy binds and activates free monomeric Cdk1 independently of protein synthesis; leading to meiosis resumption (Ferby *et al.* 1999a, Lenormand *et al.* 1999). Reversely, the injection of antisense oligonucleotides directed against its mRNA delays Cdk1 activation but does not block the process (Ferby *et al.* 1999a, Lenormand *et al.* 1999).

Therefore, injecting any of these proteins (Cyclins, Mos and Ringo) induces Cdk1 activation independently of progesterone stimulation and of protein synthesis, whereas the ablation of only one of these proteins using pharmacological inhibitors or antisense oligonucleotides does not impair meiosis resumption. These results strongly support the view that these pathways act redundantly to activate Cdk1. Accordingly, it was shown that neither progesterone nor PKI triggered GVBD and Cdk1 activation when the synthesis of Cyclin B together with Mos was prevented using a cocktail of specific antisense oligonucleotides directed against both mRNA (Haccard *et al.* 2006). The replenishment of one of these two pathways, by injecting either recombinant Cyclin B or Mos, was able to rescue Cdk1 activation induced by progesterone or PKI (Haccard *et al.* 2006). Therefore, progesterone launches more than one signalling pathway, each able to trigger meiosis resumption. The activation of at least one of these pathways, controlled either by Cyclin B or by Mos, is sufficient for the entry in MI. Moreover, since inhibiting both the synthesis of Cyclin B and Mos is sufficient for preventing Cdk1 activation, this further suggests that Speedy/Ringo could not play a critical physiological role on its own to support a key transduction pathway induced by progesterone and leading to Cdk1 activation (Haccard *et al.* 2006).

3. PP1

The activation of the phosphatase PP1 was shown to be required for meiosis resumption of *Xenopus* oocytes (Huchon *et al.* 1981b, Foulkes *et al.* 1982). The injection of specific proteic PP1 inhibitors, the Inhibitor 1 or the Inhibitor 2, prevents meiosis resumption induced either by progesterone or following the injection of PKI (Huchon *et al.* 1981b, Foulkes *et al.* 1982). Since injected Inhibitor 1 is unable to abolish meiosis resumption following the transfer of cytoplasm from a MII-arrested oocyte (Huchon *et al.* 1981b), PP1 activation is unlikely to be required within the MPF autoamplification loop. In addition, the injection of Inhibitor 1 three hours after hormonal treatment does not affect meiotic maturation. Therefore, the activation of PP1 is required at a quite early step of the signalling pathway induced by progesterone, downstream PKA inhibition. The mechanisms controlling PP1 activation as well as the molecular cascade connecting PP1 to Cdk1 activation have not been yet elucidated. Interestingly, PP1 is involved in regulating the dephosphorylation of Cdc25C at S287, at least in *Xenopus* egg extracts (Margolis *et al.* 2003, Margolis *et al.* 2006b). It would be interesting to determine whether the activation of PP1 could trigger a partial activation of Cdc25C, thus helping to switch the balance of activities between Myt1 and Cdc25C.

iv. The two-steps mechanism of Cdk1 activation

From all the experiments described above, a model involving a two-steps mechanism for Cdk1 activation has been proposed in *Xenopus* oocyte. During the first step, progesterone induces a drop of cAMP level, the subsequent downregulation of PKA activity and the synthesis of new proteins from maternal mRNA. This first part of the signalling pathway takes from 2 to 6 hours to be implemented, depending on the female and generates a starter amount of active Cdk1. This small amount of active Cdk1 then launches the second step necessary for the full activation of Cdk1, the MPF autoamplification loop, which acts as an OFF-ON switch and drives irreversibly the oocyte in M-phase. The MPF autoamplification relies on phosphorylation/dephosphorylation events and converts the stockpile of inactive pre-MPF into active MPF. The transfer of cytoplasm from a MII-arrested oocyte into a prophase-arrested oocyte mimics the autoamplification loop, as it allows a small amount of ectopic active MPF from the donor cell to launch the loop converting pre-MPF into MPF in the recipient oocyte. Cytoplasmic transfer or injection of okadaic acid, a strong inhibitor of PP1 and PP2A phosphatases, induce GVBD in the absence of protein synthesis (CHX) or in the presence of elevated levels of cAMP (IBMX) (Wasserman *et al.* 1975, Rime *et al.* 1990, Dupre *et al.* 2013). This means that the MPF autoamplification loop is independent of cAMP level, PKA activity and protein synthesis.

This model involving a biphasic activation of Cdk1 is however difficult to study and remains to be formally proved. Indeed, the induction of GVBD in response to progesterone is not totally synchronous over time in *Xenopus* oocytes and at the time of $GVBD_{50}$ (when 50% of the oocytes exhibit GVBD), some oocytes have already reached GVBD whereas some others are still defective for Cdk1 activity. Secondly, there is no morphological criterion to detect the 1st activation of Cdk1. The only morphological change detectable is the appearance of the spot that characterizes GVBD, which is induced by the full activation of Cdk1 and the establishment of the MPF automplification loop. Indeed, Cdk1 activation is initiated before the appearance of the spot. Finally, the transition between the 1st activation of Cdk1 and the MPF autoamplification loop is very fast and seems to occur without distinguishable intermediate step.

One strategy to address the role of one particular protein within the mechanism of Cdk1 activation has been to analyse whether the candidate protein can induce meiosis resumption independently or not of PKA activity and/or protein synthesis. However, although some proteins involved in Cdk1 activation such as Mos, Cyclin B and Cdc25C can trigger meiosis resumption in the presence of CHX (Gautier *et al.* 1991, Roy *et al.* 1991, Lee *et al.* 1992, Huchon *et al.* 1993b, Rime *et al.* 1994), these actors are all unable to promote meiosis resumption in the presence of a high level of PKA activity (Rime *et al.* 1992a, Matten *et al.* 1994, Rime *et al.* 1994, Dupre *et al.* 2013). This suggests that the activity of PKA locks Cdk1 activation at two points of meiosis resumption, once in the early signalling pathway, as already described, and the second time within the MPF autoamplification loop (**Fig. 16 and 24**).

1. Formation of the starter amount of active Cdk1

In response to progesterone, the starter amount of active Cdk1 can be formed by two non-exclusive means: (i) the association of newly synthesized Cyclin B with the monomeric form of Cdk1 and (ii) the conversion of a small amount of pre-MPF into active MPF by switching the balance of activities between Cdc25C and Myt1 (**Fig. 17**). This latter mechanism therefore implies that the activity of Cdc25C and/or of Myt1 must be at least partially regulated upon progesterone stimulation and independently of Cdk1 activity.

▪ *From monomeric Cdk1: the synthesis of Cyclins B*

Progesterone stimulates the basal synthesis rate of Cyclin B1, and probably B4, independently of MPF activation (Frank-Vaillant *et al.* 1999, Hochegger *et al.* 2001). These newly synthesized Cyclin B molecules then associate with monomeric Cdk1. A part of Cdk1 is already phosphorylated at T161 by

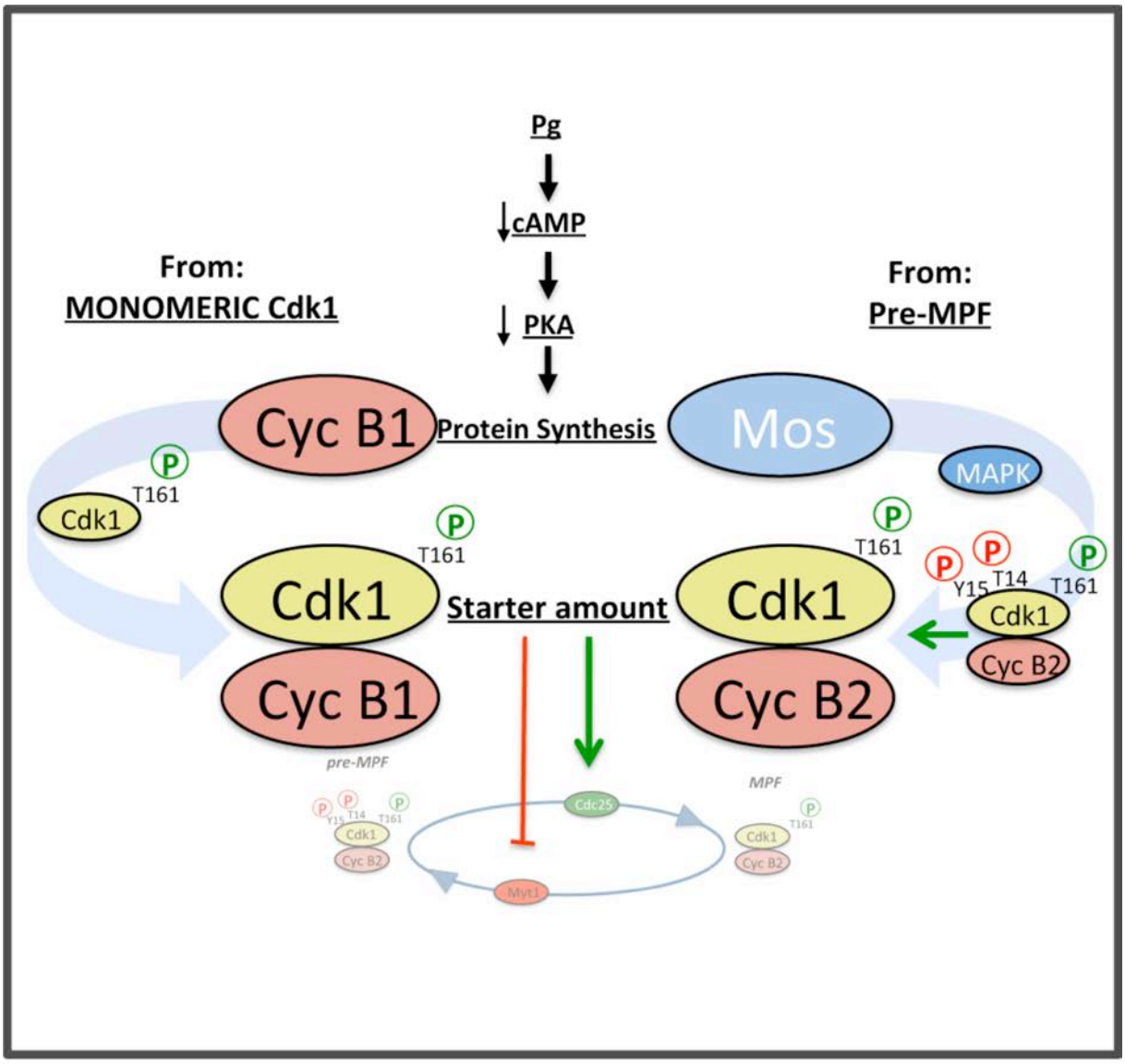

Fig. 17: Formation of the starter amount of active Cdk1

Downstream protein synthesis, two parallel and non-exclusive pathways are involved in the activation of MPF. Firstly, monomeric Cdk1 associates with newly synthetized Cyclin B1 to form a starter amount of active. Secondly, newly synthetized Mos activates the MAPK signalling that regulates the balance of activities between Cdc25 and Myt1, leading to the conversion of a small population of inactive pre-MPF in active MPF. The activation of one of these pathways is sufficient to trigger the MPF automplification loop and meiosis resumption.

CAK in prophase (De Smedt *et al.* 2002) (**Fig. 17**). In agreement with this hypothesis, the injection of a dominant negative form of Cdk1, the mutant K33R-Cdk1, which binds to Cyclin B1 and B2 in *Xenopus*, prevents meiosis resumption in response to progesterone (Nebreda *et al.* 1995). This mutated form of Cdk1 locks the activation of Cdk1 induced by Cyclin A. Importantly, it has no effect on Cdk1activation induced by the addition of okadaic acid in extracts produced from prophase oocytes, a process known to promote MPF autoamplification independently of protein synthesis (Nebreda *et al.* 1995). This result indicates that the K33R mutant of Cdk1 acts as a dominant negative form over endogenous Cdk1 by sequestering newly synthesized proteins, namely Cyclins, which are required for meiotic maturation. However, K33R-Cdk1 is unable to inhibit meiosis resumption when this process is initiated by launching the MPF autoamplification loop, suggesting that Cyclin B synthesis is unlikely required for the second step of Cdk1 auto-amplification.

One hypothesis explaining the initial activation of Cdk1 is that newly synthesized Cyclin B or injected Cyclins associate with free T161-phosphorylated Cdk1 and directly generate newly active Cdk1-Cyclin B complexes that escape Myt1 inhibition. The increasing quantity of these Cdk1-Cyclin B complexes could titrate the activity of Myt1 thus leading to a sufficient amount of active Cdk1-Cyclin B able to partly inhibit Myt1 activity (Gaffre *et al.* 2011). Accordingly, although Myt1 inhibition occurs before GVBD and Cdc25C activation, this process is no longer observed when oocytes are previously injected with Cyclin B antisense oligonucleotides (Gaffre *et al.* 2011). These results strongly argue that Myt1 inhibition depends on the newly formed active Cdk1-Cyclin B complexes and, thus, contributes to launch the MPF autoamplification loop. Another hypothesis would be a role for Ringo/speedy. Since overexpressed Ringo was shown to bind Cdk1 and Myt1 together, Ringo could help in inhibiting Myt1 either directly or by forming active Cdk1-Ringo complexes (Ferby *et al.* 1999b, Ruiz *et al.* 2008).

- ***From the pre-MPF : the synthesis of Mos, Myt1 and Cdc25***

Recombinant Mos is a much less efficient inducer of meiotic maturation compared to recombinant Cyclin B. Its ability to activate Cdk1 independently of protein synthesis relies on the amount of the injected recombinant protein and on the presence of sub-liminal amounts of progesterone (Yew *et al.* 1992). Moreover, Mos-induced Cdk1 activation totally depends on the activation of MAPK (Gross *et al.* 2000), and induces GVBD in the presence of antisense oligonucleotides against Cyclin B, meaning in the absence of Cyclin B synthesis and in the absence of newly formed Cdk1-Cyclin B complexes (Haccard *et al.* 2006). Altogether, these results strongly argue that the effect of the Mos/MAPK pathway in triggering meiosis resumption is mediated through the activation of the pre-MPF (**Fig. 17**). This function can be exerted either by inhibiting Myt1 or by activating Cdc25.

Myt1 was shown to be hyperphosphorylated and inhibited in response to progesterone before GVBD and before the activation of Cdc25 (Gaffre *et al.* 2011). The Mos/MAPK pathway is able to inhibit Myt1 through Mos, active MAPK and $p90^{Rsk}$ (Palmer *et al.* 1998, Peter *et al.* 2002, Gaffre *et al.* 2011). Accordingly, recombinant Mos leads to Myt1 partial phosphorylation in $p21^{Cip1}$-injected oocytes despite the lack of Cdk1-Cyclin B activity (Gaffre *et al.* 2011), suggesting that this pathway is sufficient to partially inhibit Myt1. However, the full phosphorylation of Myt1 in response to progesterone is still observed in Mos morpholinos-injected oocytes further treated with U0126 to specifically block the MAPK-independent activation of $p90^{Rsk}$ (Dupre *et al.* 2002, Gaffre *et al.* 2011). The Mos/MAPK pathway may therefore enhance Myt1 inhibition in response to progesterone but would not be necessary, since Myt1 complete phosphorylation is occurring also in its absence. As Cdk1 contained in pre-MPF molecules is already phosphorylated at Y15 and T14 and that these phosphorylations are not subjected to any turn-over, the key reaction to activate pre-MPF is the dephosphorylation of both inhibitory residues of Cdk1, i.e. the activation of Cdc25. Therefore, the precocious activation of Cdc25C could also generate few active MPF molecules from the huge pre-MPF store. The Mos/MAPK pathway has been shown to enhance Cdc25C activity by phosphorylation (Wang *et al.* 2010). Whether this mechanism is functional and sufficient to trigger meiosis resumption is unknown.

Importantly also, the stability of the Mos protein depends on its Cdk1-phosphorylation (Castro *et al.* 2001b). Mos accumulates at detectable levels around the time of GVBD, where it activates the MAPK pathway. This is consistent with the presence of active Cdk1, able to stabilize Mos. Moreover, Mos protein cannot accumulate in response to progesterone in oocytes where Cdk1 activation is prevented by $p21^{Cip1}$ protein (Wang *et al.* 2010). These results imply that Mos is rather dependent on Cdk1 than the opposite. Therefore, the Mos/MAPK pathway is more likely involved within the MPF automplification loop rather than in firing the system by the production of a starter amount of Cdk1 activity.

2. The MPF autoamplification loop

Once the starter amount of active Cdk1 is generated, it activates by phosphorylation many parallel pathways that quickly converge to convert the pre-MPF into active MPF. More MPF is produced from pre-MPF, more the parallel pathways are recruited, and faster is the pre-MPF converted into MPF. This MPF autoamplification reinforces the activity of Cdk1 and inhibits the Cdk1-counteracting phosphatases as in mitosis, allowing the phosphorylation of mitotic substrates. This positive feedback loop is controlled by multiple kinases: Cdk1 itself but also the Mos/MAPK pathway and the kinase Plx1; and by two main phosphatases, PP1 and PP2A.

- ***Role of Cdk1 on its regulators: the core part***

When injected in prophase, human Cdc25A induces meiosis resumption independently of progesterone and protein synthesis (Rime *et al.* 1994). Cdc25 interacts with active Cdk1-Cyclin B *in vivo* through a small region within the Cyclin B called P-Box (Jessus *et al.* 1992, Zheng *et al.* 1993). Moreover, Cdk1-Cyclin B directly phosphorylates *Xenopus* Cdc25C at T48, T67, T138, S205 and S285 and activates Cdc25C *in vitro* and *in vivo* (Hoffmann *et al.* 1993, Izumi *et al.* 1993, Strausfeld *et al.* 1994) (**Fig. 18**). As mentioned above, Cdc25C is a weak inducer of meiosis resumption in *Xenopus* due to its PKA-dependent phosphorylation at S287 (Duckworth *et al.* 2002). However, when Cdc25C is firstly *in vitro* thiophosphorylated by Cdk1-Cyclin B then injected in prophase oocytes, it becomes a potent inducer of Cdk1 activation (Hoffmann *et al.* 1993), indicating that the Cdk1-dependent phosphorylation of Cdc25C is dominant over the PKA-dependent phosphorylation at S287.

In addition to Cdc25, Myt1 is hyperphosphorylated during meiosis resumption (Mueller *et al.* 1995b, Liu *et al.* 1999, Wells *et al.* 1999). The phosphorylation of Myt1 by Cdk1-Cyclin B complexes does not really affect its activity *in vitro* (Mueller *et al.* 1995b, Booher *et al.* 1997). However, the injection of Cyclin B in the presence of CHX induces the full phosphorylation of Myt1 (Gaffre *et al.* 2011), supporting the view that the full inhibition of Myt1 is predominantly under the control of active Cdk1-Cyclin B.

Hence, it is clear that the direct phosphorylation of Myt1, which inhibits its activity, and of Cdc25, which activates this phosphatase, by Cdk1-Cyclin B constitutes the minimal core of the MPF auto-amplification loop (**Fig. 18**).

- ***Cdc25 regulation by Plx1***

In *Xenopus* egg extracts depleted of Cdk1 and Cdk2 and treated with microcystine, a PP1 and PP2A phosphatase inhibitor, Cdc25C remains phosphorylated at the time of M-phase entry, indicating that a kinase distinct from CDK contributes to Cdc25 phosphorylation (Izumi *et al.* 1995). Based on this observation, this kinase was isolated and identified as *Xenopus* Plx1, the homolog of Plk1 in mammals, as the kinase phosphorylating Cdc25C independently of Cdk1 *in vitro* (Kumagai *et al.* 1996). During meiotic maturation, Plx1 is phosphorylated and activated concomitantly with both Cdc25 and Cdk1 (Qian *et al.* 1998). The injection of $p21^{Cip1}$ abolishes Plx1 phosphorylation in response to progesterone (Karaiskou *et al.* 1998), showing that Plx1 activation depends on active Cdk1. Accordingly, active Cdk1-Cyclin B complexes phosphorylate directly Plx1 at S340, a process responsible for its electrophoretic retardation in M-phase (Kelm *et al.* 2002) (**Fig. 19**). Plx1 is therefore implicated within the MPF autoamplification, *i.e*. activated by Cdk1 and contributing to Cdk1 activation.

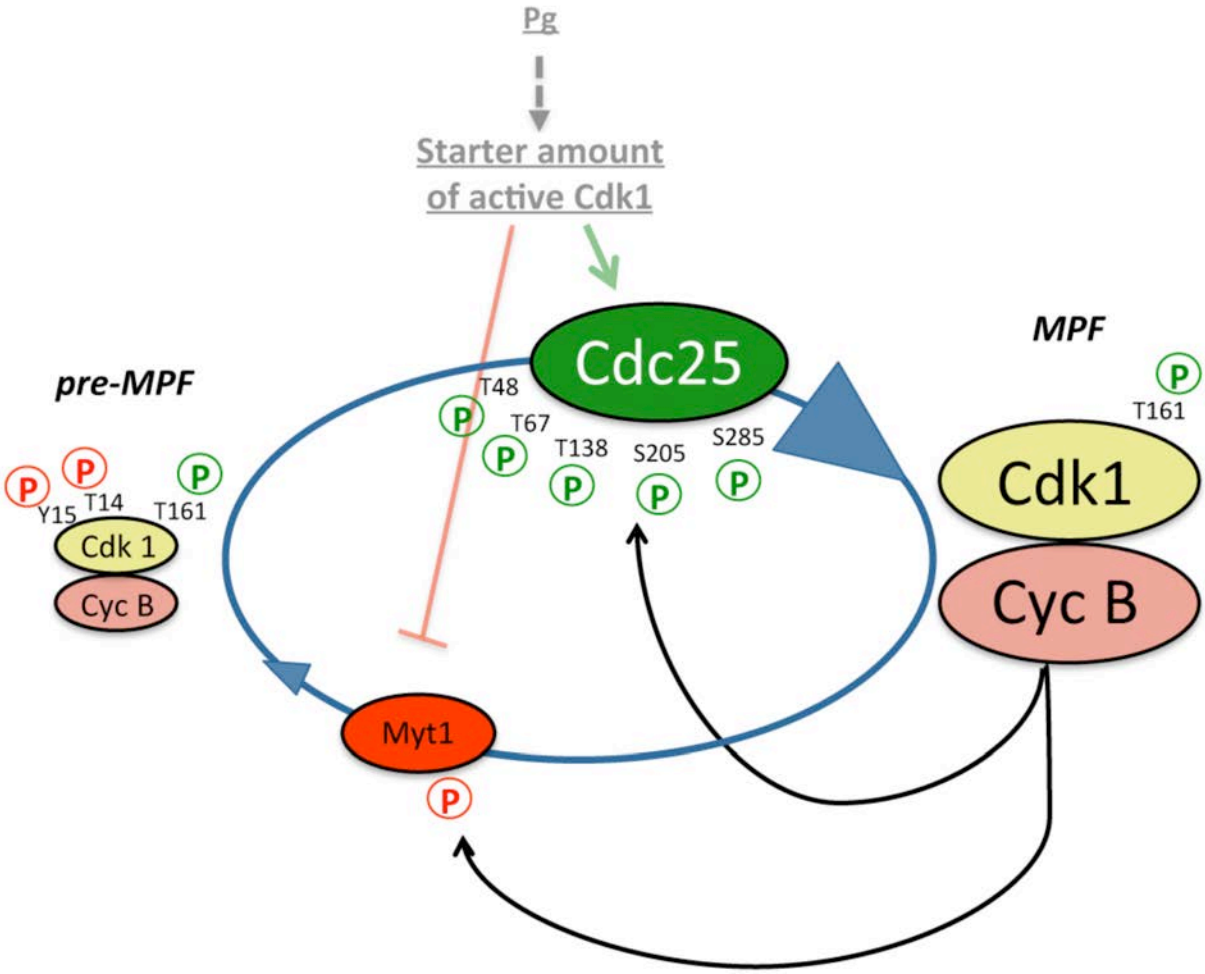

Fig. 18: MPF regulates its direct regulators

Active MPF directly phosphorylates its own regulators Cdc25 and Myt1. Cdc25 phosphorylation by Cdk1 promotes its full activation while the Cdk1-dependent phosphorylation of Myt1 inhibits its activity. This positive effect of Cdk1 on its own regulators represents the core part of the MPF auto-amplification loop.

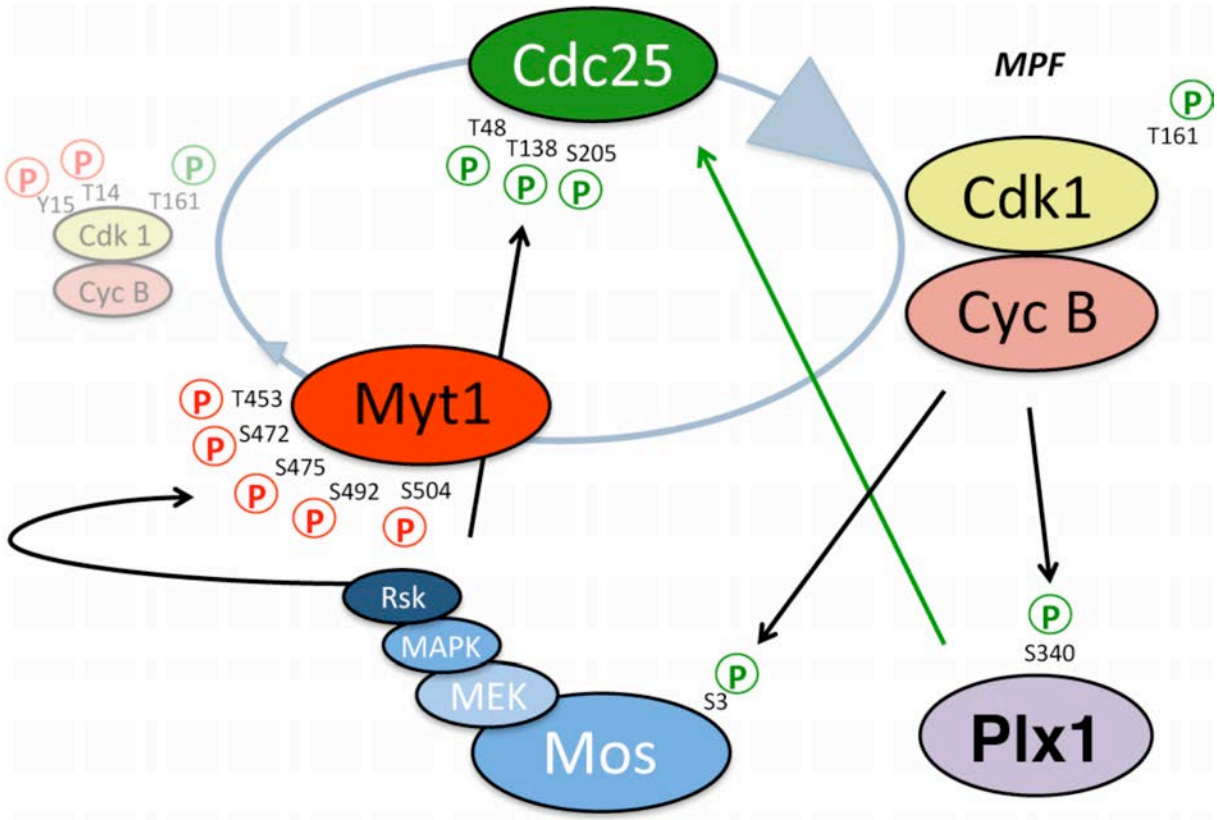

Fig. 19: The role of Plx1 and of the Mos/MAPK pathway in the MPF autoamplification loop

Plx1 and the Mos/MAPK pathway are activated downstream the starter amount of active Cdk1. The Mos/MAPK pathway simultaneously contributes to Cdc25 activation and Myt1 inhibition. The Cdk1-dependent activation of Plx1 is further required for the full activation of Cdc25.

The role of Plx1 within the MPF autoamplification loop is to facilitate Cdc25C activation, hence contributing indirectly to Cdk1 activation. The overexpression of a constitutive form of Plx1 induces meiotic maturation independently of progesterone addition whereas its inhibition using specific antibodies delays, but does not block, meiosis resumption induced by either progesterone or recombinant Cdc25C (Qian *et al.* 1998). Plx1 is therefore sufficient but not necessary for the activation of Cdk1, suggesting that other kinases are implicated in the process of Cdc25C activation. Plx family comprises three members, Plx1, Plx2 and Plx3, opening the possibility that Plx2 and Plx3 could participate to the autoamplification loop. Plx2 and Plx3 are expressed in *Xenopus* oocytes and are concomitantly activated with Plx1 and Cdk1 (Duncan *et al.* 2001). As for Plx1, their overexpression stimulates progesterone-induced oocyte maturation whereas overexpressing catalytically inactive kinases delays meiosis resumption (Duncan *et al.* 2001). This suggests that, at least in oocytes, these three Plx isoforms may be regulated similarly and have certainly redundant functions.

The role of Plx1 in regulating Cdc25C was deciphered in two types of extracts prepared from prophase-arrested oocytes, either “diluted” or “undiluted”. The “undiluted” extracts do not contain any membrane but support protein synthesis and the addition of PKI activates Cdk1 (Qian *et al.* 2001). Immunodepleting Plx1 from these extracts prevents the activation of both Cdc25C and Cdk1 suggesting that Plx1 contributes to Cdk1 activation (Qian *et al.* 2001). In diluted extracts, the addition of Cdc25A produces a linear activation of Cdk1 that can be visualized by the progressive dephosphorylation of Cdk1 at Y15 in parallel with the progressive phosphorylation of Cdc25 (Karaiskou *et al.* 1999). This slow increase of both Cdk1 and Cdc25 activities results from the direct dephosphorylation of Cdk1 by Cdc25, in a simple two-components system, that is independent of Plx1. This linear activation of Cdk1 does not represent the OFF-ON switch occurring *in vivo.* This latter phenomenon is however reproduced *in vitro* in “diluted” extracts after the addition of okadaic acid to inhibit PP2A/PP1 activity (Karaiskou *et al.* 1998). Okadaic acid leads to the abrupt activation of Cdk1, consequential to Cdc25 hyperphosphorylation and the full dephosphorylation of Cdk1 at Y15 (Karaiskou *et al.* 1999). The depletion of Plx1 from these extracts abolishes this fast activation of Cdk1 in response to okadaic acid (Karaiskou *et al.* 1999), supporting the role of Plx1 within the MPF autoamplification loop by activating Cdc25C (**Fig. 19**).

- ***The Mos/MAPK pathway***

At the time of GVBD, the Cdk1-dependent phosphorylation of Mos at S3 stabilizes the protein and allows its accumulation (Castro *et al.* 2001b). The Mos/MAPK pathway reinforces the MPF autoamplification loop, as discussed before. Indeed, inhibiting the Mos/MAPK pathway with U0126

leads only to a partial activation of Cdk1 in oocytes stimulated by progesterone (Gross *et al.* 2000). As described before, the Mos-MAPK pathway has the double potential to act on both Cdc25 and Myt1 (**Fig. 19**).

– Effects on Cdc25

In mitotic *Xenopus* extracts, MAPK phosphorylates Cdc25C at T48, T138 and S205 independently of Cdk1 and Plx1, but without producing the characteristic shift in its electrophoretic migration detected in M-phase (Wang *et al.* 2007) (**Fig. 19**). This shows that, even though MAPK phosphorylates Cdc25, it is not the only kinase involved in this process. To measure the phosphatase activity of Cdc25 before and after phosphorylation, its ability to convert pre-MPF in MPF was assayed using an *in vitro* H1 kinase assay. Purified pre-MPF is not active and therefore cannot phosphorylate H1 in an *in vitro* kinase assay. The addition of Cdc25 promotes the conversion of pre-MPF into MPF, promoting H1 phosphorylation. It was shown that in order to reach the same level of phosphate incorporation within H1, a lower concentration of Cdc25 was required if the protein was previously *in vitro* phosphorylated by MAPK. These results showed that the MAPK-dependent phosphorylation of Cdc25 increases Cdc25 ability to convert pre-MPF in MPF. More recently, it was also shown that the kinase $p90^{Rsk2}$ is also able to directly phosphorylate Cdc25 (Wang *et al.* 2010).

– Effect on Myt1

In addition to Cdc25, $p90^{Rsk}$ interacts, phosphorylates and inhibits Myt1 (Palmer *et al.* 1998). Using again the *in vitro* H1 kinase assay to measure Cdk1 activation, it was shown that active Myt1 kinase, immunopurified from prophase-arrested oocytes, is able to inhibit Cdk1-Cyclin B activity. However, when Myt1 is previously incubated with active $p90^{rsk}$, its inhibitory activity towards Cdk1-Cyclin B is reduced (Palmer *et al.* 1998). $p90^{rsk}$ phosphorylates Myt1 at 5 different sites (T453, S472, S475, S492 and S504), which are different from the ones targeted by Cdk1 (Ruiz *et al.* 2010)(**Fig. 19**). Using *in vitro* H1 kinase assay, it was shown that wild type Myt1, as well as the mutant Myt1-5A (without MAPK phosphorylation sites) and the mutant Myt1-3A (without Cdk1 phosphorylation sites) are all able to efficiently inhibit Cdk1 activity (Ruiz *et al.* 2010). However, if wild type Myt is previously incubated with either Cdk1-RINGO or $p90^{Rsk}$, its ability to block H1 activity in this *in vitro* kinase assay is reduced (Ruiz *et al.* 2010). Therefore both Cdk1 and Mos/MAPK/$p90^{Rsk}$ are able to inhibit Myt1 activity. Interestingly, Cdk1-RINGO is able to inhibit Myt1-5A, and conversely $p90^{Rsk}$ is able to block Myt1-3A (Ruiz *et al.* 2010). These results show that either Mos/MAPK/$p90^{Rsk}$ or Cdk1-RINGO activities phosphorylate distinct sites on Myt1 and have the potential to inhibit Myt1. On the other hand, none of these two convergent redundant pathways is necessary for the inhibition of Myt1.

- ***Greatwall and PP2A-B55δ***

As in mitosis, the phosphatase activities counteracting Cdk1 activity needs to be inhibited to resume meiosis. This shut off is required to allow the phosphorylation of regulators of Cdk1 activity (as Plx1, Cdc25, Myt1, MAPK, *etc*) as well as substrates of Cdk1 necessary for the mechanistic events of cell division (as lamins, condensins, histones, MAPs, *etc*).

The inhibition of PP2A/PP1 by injecting okadaic acid is sufficient to induce meiotic maturation in *Xenopus* oocytes, independently of progesterone, of PKA downregulation and of protein synthesis (Rime *et al.* 1990). In prophase diluted extracts, the addition of OA also leads to Cdk1 activation (Karaiskou *et al.* 1998, Karaiskou *et al.* 1999). This indicates that PP2A inhibition is mandatory for the MPF autoamplification to run. Since PP2A activity counteracts the Cdk1-dependent phosphorylations, its inhibition is necessary to stabilize the Cdk1-dependent phosphorylations of Cdc25C and Myt1, which represents the core part of the MPF autoamplification loop, and probably Cdk1 phosphorylation of other players of the loop, as Plx1 and members of the Mos/MAPK pathway. In addition, Cdk1 phosphorylates a lot of mitotic substrates to orchestrate M-phase progression and these phosphorylation events are only stable if the activity of PP2A is inhibited (Castilho *et al.* 2009, Mochida *et al.* 2009, Vigneron *et al.* 2009, Burgess *et al.* 2010, Goldberg 2010).

As in mitosis, the kinase responsible for the inhibition of PP2A is Greatwall (Gwl) (Castilho *et al.* 2009, Vigneron *et al.* 2009, Burgess *et al.* 2010, Goldberg 2010). The search of the substrate of Gwl led to the identification of Arpp19, a small protein of the endosulfine family (Gharbi-Ayachi *et al.* 2010, Mochida *et al.* 2010). Arpp19 is phosphorylated at S67 by Gwl, which is necessary and sufficient to convert Arpp19 in a powerful inhibitor of a specific isoform of PP2A, PP2A-B55δ (Gharbi-Ayachi *et al.* 2010, Mochida *et al.* 2010) (See chapter: I-A.e.ii.2. “role of proteins phosphatase”) **(Fig. 10)**. The endosulfine family comprises two classes of proteins, which are encoded by different genes: the endosulfines (α and β) and the cAMP-phosphoregulated proteins (Arpp).

In *Xenopus* oocytes, the injection of a purified active form of Gwl or of an active mutant of Gwl, the K71M-Gwl, promotes Cdk1 activation independently of progesterone (Yamamoto *et al.* 2011, Dupre *et al.* 2013). Gwl activates Cdk1 through the phosphorylation of Arpp19 at S67 since the injection of a non-phosphorylatable form of Arpp19 at S67 (Arpp19-S67A) abolishes Cdk1 activation induced by K71M-Gwl (Dupre *et al.* 2013). The Arpp19-S67A mutant is able to block meiosis resumption in response to progesterone but also in response to the transfer of cytoplasm from a MII-arrested oocytes (Dupre *et al.* 2013). This demonstrates that the Gwl/Arpp19/PP2A module plays a critical role in the MPF automplification loop. Importantly, Cdk1 activity is required for the activation of the Gwl-ARPP19 pathway (Dupre *et al.* 2013). This reinforces the view that Gwl/Arpp19/PP2A is not part of the first step of Cdk1 activation, where the starter amount of Cdk1 activity is generated from

scratch, but is embarked into the MPF autoamplification loop that is launched by the first active molecules of Cdk1. Once Gwl is activated, Arpp19 interacts with PP2A-B55δ and inhibits its activity (Dupre *et al.* 2013). This result was further confirmed using a S67-thiophosphorylated form of ARPP19. When injected in prophase, this phosphorylated form of Arpp19 interacts with PP2A-B55δ independently of protein synthesis, of Cdk1 activation and of PKA downregulation and promotes the activation of Cdk1 independently of PKA activity (Dupre *et al.* 2013)

The role of Gwl-ARPP19 is conserved among species but seems to be differentially regulated. In *Drosophila*, oocytes depleted for *endo* (the unique member of the ARPP19/Endosulfine family) are unable to progress through meiosis because of a low level of Cdk1 substrate phosphorylation, even in the presence of high Cdk1 activity (Von Stetina *et al.* 2008). In mouse, siRNA for both Endosulfine/Arpp19 abolishes meiosis resumption (Matthews *et al.* 2014), whereas the depletion of Gwl only affects the progression in meiosis after meiosis I (Adhikari *et al.* 2014). These observations suggest that another parallel pathway is activated in order to inhibit PP2A-B55δ activity in these cells, which depends on Arpp19 but not on Gwl. An explanation about this unknown pathway was provided by studies in starfish oocytes. In the oocytes of this species, Gwl is nuclear while Arpp19 is cytoplasmic (Hara *et al.* 2012). When Gwl is removed by enucleating the oocytes or inhibited by injecting blocking antibodies, Cdk1 is still activated (Hara *et al.* 2012). However, the depletion of Arpp19 abolishes Cdk1 activation, in the presence or in the absence of Gwl (Okumura *et al.* 2014). This effect is due to the phosphorylation of Arpp19 by Cdk1, at S69, a site distinct from the residue targeted by Gwl (S106 in starfish). This phosphorylation by Cdk1 allows Arpp19 to bind and to inhibit PP2A with a lower efficiency compared to ARPP19 phosphorylated by Gwl (Okumura *et al.* 2014). Whether this mechanism is conserved in *Xenopus* oocytes or in other systems is not known.

e. The MI-MII transition

In *Xenopus* oocytes, the metaphase I spindle organizes 30 minutes after GVBD. After anaphase I and then the extrusion of the 1st polar body, the metaphase II spindle organizes at the cortex and the oocyte arrests at this stage. This MI-MII transition lasts 120 minutes starting from GVBD until the MII arrest. It is characterized by the decrease of 50%-70% of Cdk1 activity that occurs during anaphase I. However, this drop in Cdk1 activity does not reach the basic levels assayed in prophase. Thereafter, the re-increase of Cdk1 activity, allows the formation of the metaphase II spindle. During this transition, Cdk1 activity is proportional to the concentration of Cyclin B, which is positively regulated by MAPK (See chapter: I-B.e.ii. “Function of the Mos/MAPK pathway”) and negatively controlled by APC^{Fizzy} (Cdc20). When the 1st meiotic division is completed, the nuclear envelope does not assemble, chromosomes remain condensed and DNA replication is prohibited (Huchon *et al.* 1981a, Gerhart *et*

al. 1984, Gard 1992, Furuno *et al.* 1994). All these events depends on the activity of Cdk1 and by the Mos/MAPK pathway.

The transition from metaphase I to anaphase I as well as the extrusion of the 1st polar body rely on the degradation of Cyclin B that leads to the decrease of Cdk1 activity. After metaphase I, all the isoforms of Cyclins (B1, B2, B4 and B5) are ubiquitinated by APC^{Cdc20} (APC^{Fizzy} in *Xenopus*) and degraded by the proteasome (Glotzer *et al.* 1991, Kobayashi *et al.* 1991, Hochegger *et al.* 2001, Taieb *et al.* 2001). The activation of APC^{Cdc20}, which correlates with the phosphorylation status of Cdc27, a subunit of APC (King *et al.* 1995), is absolutely required in *Xenopus* extracts to produce cyclical oscillation of Cdk1 activity (Lorca *et al.* 1998). Surprisingly, both the APC^{cdc20} activation and the Cyclin B degradation, were shown to be dispensable for the entry in MII. The injection of antisense against Cdc20 completely blocks the degradation of Cyclin B, as well as the Cdk1 partial inactivation characteristic of the MI-MII transition (Taieb *et al.* 2001). Similar results were obtained by inactivating the APC by injecting blocking-antibodies against Cdc20 or Cdc27 or by injecting Mad2, an APC inhibitor (Peter *et al.* 2001). In all cases, oocytes extrude the 1st polar body, reaccumulate Cyclin E and are able to enter in MII as seen by the assembly of a MII spindle (Peter *et al.* 2001, Taieb *et al.* 2001). These results suggest that the activation of APC^{Cdc20} is not required for the segregation of homologous chromosome as it is for the segregation of sister chromatids. However, the injection of undegradable Cyclin B at GVBD was shown to prevent anaphase I, leading to abnormal spindles not anchored to the cortex and failing to segregate chromosomes (Huchon *et al.* 1993a). this result suggests that the absence of Cyclin B degradation and thus of Cdk1 activity drop prevents the exit from the 1st meiotic division. In a similar way, the expression of the APC inhibitor Erp1 arrests oocytes in MI, without the extrusion of the 1st polar body, demonstrating the requirement of APC activation for the MI-MII transition (Ohe *et al.* 2007, Tung *et al.* 2007). One explanation for these discrepancies would be the incomplete inhibition of APC by antisense against Cdc20 and by blocking antibody against Cdc27, which would leave a population of APC active to promote the degradation of securine and a local inhibition of Cdk1. Another hypothesis could be the involvement of Cdh1 during the MI-MII transition, a role that has not yet been investigated in *Xenopus*.

i. The role of Cdk1

The activation of the APC during this transition is under the control of Cdk1 since the injection of $p21^{Cip1}$ at GVBD completely blocks Cdc27 phosphorylation and delays Cyclin B degradation (Frank-Vaillant *et al.* 2001). Therefore, Cdk1 is positively regulating its own inactivation by controlling Cyclin B degradation, as it is in mitosis (**Fig. 20**). While the APC has to be activated to exit from MI, it has also to be inactivated to enable the oocyte to enter in MII. The simplest explanation for APC

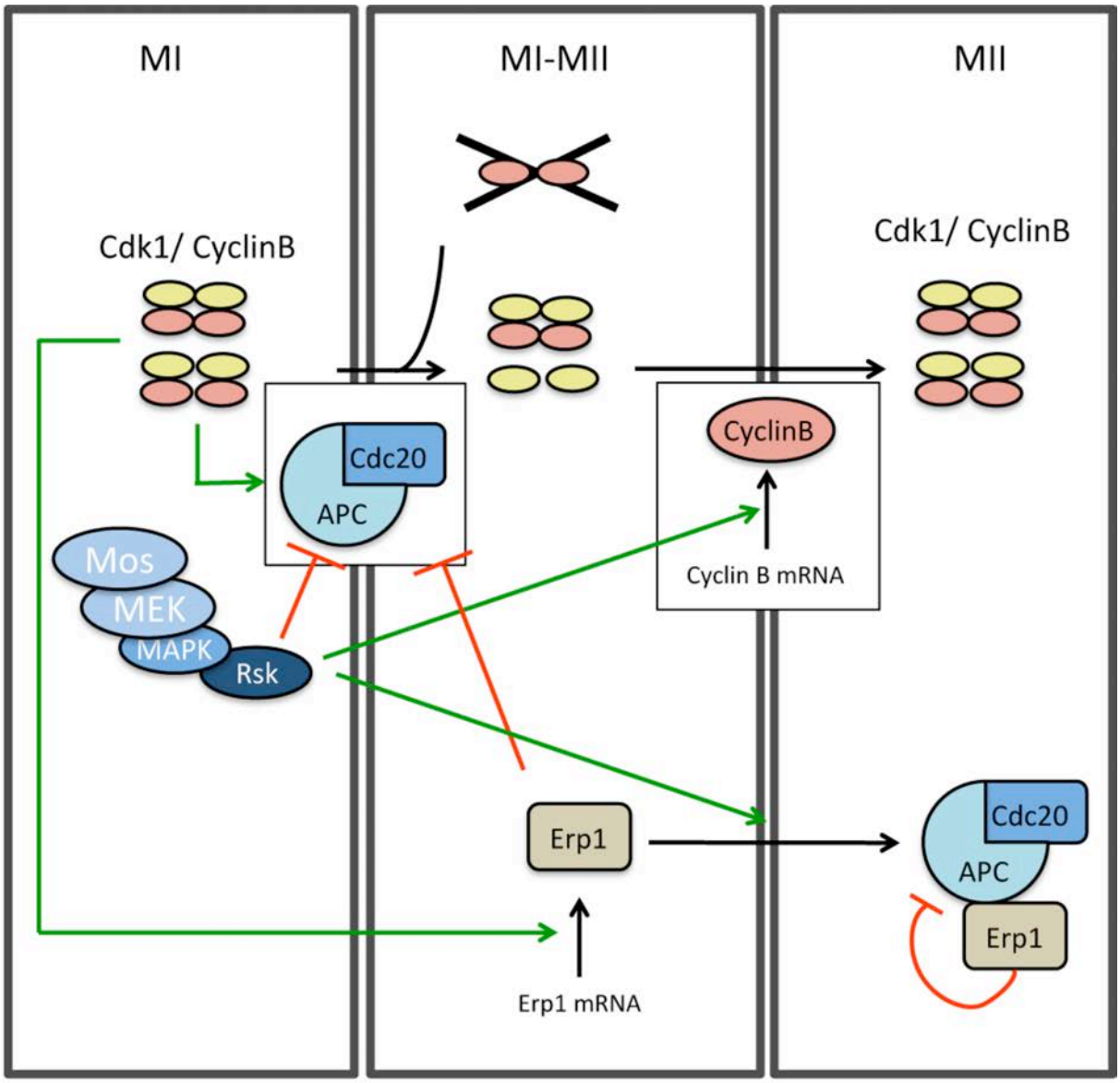

Fig. 20: The transition from the Meiosis I to the Meiosis II

The activity of Cdk1 and the Mos/MAPK pathway cooperate to ensure a correct M-M transition. Cdk1 promotes the activation of APC^{Cdc20}, which is responsible for Cyclin B degradation, and the translation of Erp1, which is a specific meiotic inhibitor of APC. The Mos/MAPK pathway contributes to Cdk1 re-activation by positively controlling Cyclin B turnover.

inactivation would be the loss of Cdk1 positive activity caused by the APC-induced degradation of Cyclin B. However, it was shown that APC inactivation requires the translation of a small amount of Erp1, an APC inhibitor responsible on the CSF arrest in MII (Ohe *et al.* 2007) (see chapter: I-B.f.iv. "Erp1/Emi2"). Erp1 starts to accumulate around 1 hour after GVBD at the time of Cyclin B re-accumulation. The knock-down of Erp1 blocks totally Cyclin B re-accumulation as well as Cdk1 reactivation leading to DNA replication (Ohe *et al.* 2007). The translational regulation of Erp1 is based on the polyadenylation of its mRNA that depends on Cdk1 activity and not on the Mos/MAPK pathway (Tung *et al.* 2007) (**Fig. 20**). All these data suggest that Cdk1 activity orchestrates its own inhibition by activating APC, but also facilitates its own reactivation, promoting the translation of Erp1 in order to inhibit the APC (**Fig. 20**).

ii. Function of the Mos/MAPK pathway

The accumulation of Cyclin B required to enter MII is independent of Cdk1 activity, as the injection of $p21^{Cip1}$ at GVBD does not impair Cyclin B accumulation (Frank-Vaillant *et al.* 2001). In contrast, the Mos/MAPK module plays an essential role in regulating this critical period of meiosis. The requirement of the Mos/MAPK pathway for the MI-MII transition was demonstrated using different approaches: the injection of "classical" antisense oligonucleotides against Mos mRNA or of Mos neutralizing antibodies at GVBD (Furuno *et al.* 1994), the injection of Mos-Morpholinos antisense oligonucleotides (Dupre *et al.* 2002) and the pharmacological inhibition of MAPK activation using U0126, a MEK inhibitor (Gross *et al.* 2000). Under all these conditions, Cyclin B is degraded and Cdk1 is thus inactivated, oocytes exit from meiosis I but do not enter into meiosis II. They are driven in a pseudo interphasic state with the reformation of nuclei. However, while U0126-treated oocytes remain arrested at this interphasic state, the Mos-morpholino injected oocytes slowly reaccumulate Cyclins B and reactivate Cdk1. Thereafter, the activity of Cdk1 oscillates, mimicking the Cdk1 pattern in embryonic cell divisions (Dupre *et al.* 2002).

The different effects caused by the inhibition of the Mos/MAPK pathway over Cdk1 reactivation can be explained by several reasons. The first one is that 50 µM of U0126, the concentration used to prevent MAPK activation in oocytes, inhibits other kinases that MEK that could account for the total block in interphase, as suggested in Dupre *et al.* (2002). A second hypothesis is that the Mos kinase, that is impaired by morpholino but not by U0126, has other targets than MEK that can act in the presence of U0126 but not in the presence of morpholino antisense, although this hypothesis is highly improbable. Another hypothesis relies on the activity of $p90^{Rsk}$ activity, the fourth kinase of the Mos/MEK/MAPK/$p90^{Rsk}$ cascade. While $p90^{Rsk}$ remains partially activated in Mos morpholino-injected oocytes (Dupre *et al.* 2002), its activity is totally abolished in U0126-treated oocytes (Gross *et al.*

2000). Moreover, the overexpression of a constitutively active form of $p90^{Rsk}$ in U0126-treated oocytes rescues the reaccumulation of Cyclin B, the hyperphosphorylation of Cdc27 and the formation of metaphase II spindles in the absence of detectable MAP kinase activity (Gross *et al.* 2000). These results reveal a prominent role of $p90^{Rsk}$ that could be preserved in the presence of Mos morpholino antisense but not with U0126. The effect of the Mos/MAPK module on Cyclin B accumulation can be exerted by increasing the synthesis of new Cyclin B or/and by decreasing the degradation of the protein (**Fig. 20**). When Cyclin B degradation is blocked with antisense oligos against Cdc20 mRNA, the inhibition of the Mos/MAPK pathway by U0126 still decreases the accumulation of Cyclin B (Taieb *et al.* 2001), showing that the Mos/MAPK pathway is able to positively regulate Cyclin B synthesis. Moreover, the inhibition of the Mos/MAPK module by U0126 accelerates the degradation of radiolabeled Cyclin B injected at GVBD (Gross *et al.* 2000), showing that the Mos/MAPK pathway is also downregulating Cyclin B degradation.

Therefore, the Mos/MEK/MAPK/$p90^{Rsk}$ cascade plays a critical role during the MI-MII transition by reducing the rate of Cyclin B degradation and increasing its synthesis. These two concomitant effects then contribute to attenuate Cdk1 inactivation between the two divisions and to promote its re-activation responsible for MII entry (**Fig. 20**).

iii. The molecular control of DNA replication

An important feature of the meiotic divisions is that oocytes must not replicate their genomic DNA between these two divisions. This process is essential for the success of reproduction and the embryonic development as it reduces by half the ploidy of the oocyte and leads to the formation of a haploid gamete. However, the unfertilized oocyte has to be competent for DNA replication in order to support the embryonic cell cycles following fertilization. Indeed, the embryonic genome is not expressed during the first 13 cell cycles that follow fertilization and these embryonic divisions are entirely ensured by the molecular machineries stored by the oocyte before fertilization. Therefore, oocytes must synchronize the acquisition of the competence to replicate DNA with some mechanisms preventing the usage of this competence before fertilization.

1. The competence to replicate DNA

Prophase-arrested oocytes are unable to replicate their DNA. This competence is acquired during meiotic maturation (Furuno *et al.* 1994, Lemaitre *et al.* 2002, Whitmire *et al.* 2002). This process can be investigated by inhibiting protein synthesis at different time after GVBD. The addition of CHX at GVBD promotes the formation of interphasic nuclei that are unable to replicate (Furuno *et al.* 1994).

Hence, oocytes at that stage are still incompetent to replicate. However, when CHX is added later, 45 minutes after GVBD, oocytes form nuclei that replicate DNA (Furuno *et al.* 1994). This pioneer experiment demonstrates that the competence to replicate DNA is established by the synthesis of some proteins during the 45 minutes that follow GVBD, hence at the period of the metaphase I to anaphase I transition. However, DNA replication does not take place under physiological conditions, meaning that this competence is not functional because a mechanism inhibiting DNA replication is also settled in a protein-synthesis dependent manner during the MI-MII transition.

All the proteins of the pre-RC are present at the protein level in prophase-arrested oocytes, with the exception of Cdc6 (Lemaitre *et al.* 2002, Whitmire *et al.* 2002). This protein is synthesized from maternal mRNA during meiotic maturation and starts to accumulate 45 minutes after GVBD (Lemaitre *et al.* 2002, Whitmire *et al.* 2002). Importantly, the injection of recombinant Cdc6 induces DNA replication in oocytes treated with CHX at GVBD, while inhibiting its synthesis with antisense oligonucleotides prevents DNA replication in oocytes incubated with CHX 45 minutes after GVBD (Lemaitre *et al.* 2002). Hence, Cdc6 corresponds to the protein whose synthesis starts 45 minutes after GVBD and that is required for the oocyte to be competent to replicate DNA. Interestingly, this mechanism regulating the competence to replicate DNA is conserved in other species (Lemaitre *et al.* 2004)(**Fig. 21**).

2. The inhibition of DNA replication

Several non-exclusive mechanisms can inhibit DNA replication during meiotic maturation. In prophase, pre-RCs cannot be formed because Cdc6 is missing but also because other proteins such as ORC proteins are localized within the cytoplasm or are only partly nuclear (Lemaitre *et al.* 2002). The localization of the pre-RCs components could therefore account for the inability of the oocyte to support DNA replication. However, the obvious mechanism that prevent DNA replication in prophase is the absence of Cdc6. Because this factor is missing, MCMs cannot bind on chromatin in prophase extracts, even though the nucleus/cytoplasm organization is lost and allows a direct access to chromatin (Whitmire *et al.* 2002). The absence of Cdc6 is a key mechanism to avoidthe assembly of pre-RCs onto chromatin in prophase but also during the first period of meiotic resumption, until right after GVBD. Around 45 minutes after GVBD, Cdc6 starts to accumulate and a minimal amount of Cdc6 is sufficient for the formation of the pre-RCs. This period is critical for the oocyte as the accumulation of Cdc6 starts at a period where the activity of Cdk1 decreases. This period is therefore favourable for DNA replication but two activities prevent the occurrence of DNA replication after meiosis I: Cdk1 and the Mos/MAPK pathway (**Fig. 21**).

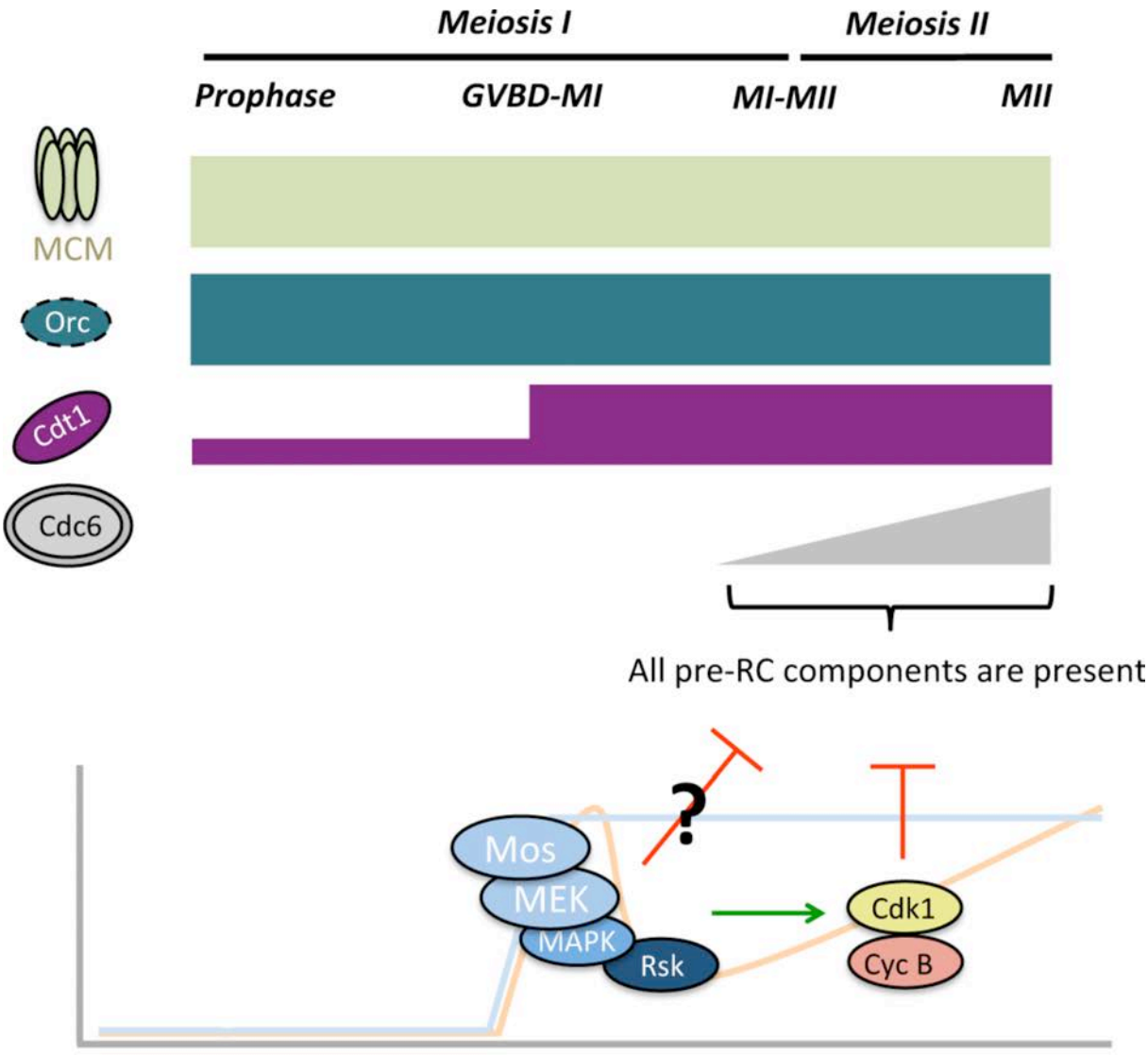

Fig. 21: The regulation of DNA replication in *Xenopus* oocytes

Xenopus oocytes acquire the competence to replicate during the MI-MII transition due to the accumulation of the replicative factor Cdc6. Once Cdc6 is present, the Pre-RC becomes functional but DNA replication does not take place to ensure the formation of a haploid gamete. This inhibition of DNA replication depends on the synergistic action of the Mos/MAPK pathway and on the residual activity of Cdk1-Cyclin B between the two meiotic divisions.

- ***Cdk1***

The role of Cdk1 activity in inhibiting DNA replication during MI-MII transition was first enlightened by injecting at GVBD the dominant negative form of Cdk1 (Cdk1-K33R), which induces DNA replication in *Xenopus* oocytes (Furuno *et al.* 1994). Furthermore, when Cdk1 reactivation is inhibited by injecting Wee1 in Metaphase I, Cdk1 is inactivated by Y15 phosphorylation and oocytes enter into a replicative state (Nakajo *et al.* 2000). This suggests that the "threshold" level of Cdk1 activity preserved during the MI-MII transition is sufficient to inhibit DNA replication at this critical period. This hypothesis was confirmed using "meiotic extracts" (M-M extracts) (Iwabuchi *et al.* 2000). These extracts are generated from MI oocytes and recapitulate all the molecular events occurring during the MI-MII transition (Iwabuchi *et al.* 2000). The addition of CHX in these extracts promotes the degradation of Cyclin B, the full inactivation of Cdk1 and DNA is replicated (Iwabuchi *et al.* 2000). The level of Cdk1 activity during the MI-MII transition is directly proportional to the concentration of Cyclin B. Hence, adding a non-destructible Cyclin B reactivates Cdk1 in a dose-dependent manner. Using this approach, it was shown that the minimal level of Cdk1 activity necessary to block the formation of nuclei and DNA replication is similar to the one measured *in vivo* (Iwabuchi *et al.* 2000). Altogether, these results indicate that as in somatic cells, Cdk1 is a potent inhibitor of DNA replication in oocyte and, more importantly, that the residual activity of Cdk1 preserved between the two meiotic divisions contributes to the inhibition of DNA replication during the MI-MII transition.

- ***The Mos/MAPK pathway***

Strikingly, inhibiting the Mos/MAPK pathway also leads to the reformation of nuclei that replicate DNA after meiosis I (Furuno *et al.* 1994, Gross *et al.* 2000, Dupre *et al.* 2002). Whether the mechanism induced by the Mos/MAPK pathway to inhibit DNA replication is direct or is mediated through Cdk1 has not been yet determined. As mentioned above, these two activities rely on each other during the MI-MII transition. Inhibiting one leads to the inactivation of the other one, rendering difficult to determine their respective roles. Since the Mos/MAPK pathway positively regulates the accumulation of Cyclin B by acting at the level of both its synthesis and its degradation, the inhibitory action of the Mos/MAPK pathway towards DNA replication can rely on its ability to maintain and reactivate Cdk1 during the MI-MII transition. Accordingly, injecting recombinant Mos is not sufficient to abolish DNA replication in oocytes treated with CHX 45 minutes after GVBD (Furuno *et al.* 1994), suggesting that the synthesis of another protein than Mos is required for the inhibition of DNA replication. This newly synthetized protein could correspond to Cyclin B (Gross *et al.* 2000, Taieb *et al.* 2001). To investigate the role of the Mos/MAPK pathway in preventing DNA replication

independently of Cdk1-Cyclin B, Cdk1 activity must be specifically inhibited at GVBD in oocytes wherein the MAPK is kept active. This could be achieved by injecting Cyclin B antisense oligonucleotides in prophase oocytes, then to induce meiotic maturation with progesterone and to inject recombinant Mos at GVBD. Another strategy will be to co-inject the dominant negative mutant Cdk1-K33R together with Mos at GVBD.

Interestingly, in starfish oocytes, oocytes do not arrest at MII, but in G_1, after the completion of the second meiotic division, and the Mos/MAPK/$p90^{Rsk}$ pathway is responsible for this arrest (Mori *et al.* 2006). During the G_1 arrest that precedes fertilization, pre-RCs are already formed onto the DNA of the female pro-nucleus but DNA replication is inhibited (Tachibana *et al.* 2010). In this model system, the activity of $p90^{Rsk}$ activity is both necessary and sufficient to prevent origin firing by blocking the loading of the protein Cdc45 on the chromatin. Under this condition, DNA polymerases cannot be recruited and as a consequence, replicative forks are not activated. The inhibition of $p90^{Rsk}$ using a blocking antibody induces the loading of Cdc45, the firing of replicative forks and DNA replication, in the absence of fertilization (Tachibana *et al.* 2010). Conversely, Cdc45 cannot be loaded onto chromatin in fertilized oocytes previously injected with a constitutively active form of $p90^{Rsk}$ (Tachibana *et al.* 2010). The ability of $p90^{Rsk}$ to inhibit DNA replication is independent of Cdk1 activity as these oocytes are arrested in G_1 with no active MPF. Similar results were further obtained in sea urchin oocytes, which are also arrested at the pronucleus stage at the end of meiotic maturation. In this species, it was further shown that the inhibition of the Mos/MAPK pathway by U0126, and the concomitant inhibition of ATM/ATR kinases using Caffeine promotes DNA replication independently of fertilization (Aze *et al.* 2010). These data suggest that S-phase is inhibited in those oocytes by the synergic action of the Mos/MAPK pathway and the checkpoint kinases ATM/ATR. Whether $p90^{Rsk}$ is the end product of the Mos/MAPK cascade that plays a specific role in inhibiting DNA replication, independently of Cdk1, in *Xenopus* oocytes deserves further investigation. However, Cdc45 is clearly the limiting step targeted by $p90^{Rsk}$ in Echinoderms, whereas this protein does not appear to be a key limiting factor in *Xenopus*.

f. The CSF arrest

i. Discovery of CSF

The pathway responsible for the second arrest can be divided into two parts: an upstream part very well conserved through evolution and a downstream part composed of effectors responsible for arresting the oocytes at different steps according the species and that vary between species. CSF was first described in Amphibians by Masui and Markert in 1971 by injecting the cytoplasm from a MII-arrested oocyte in one of the two blastomeres of a two-cell embryo (Masui *et al.* 1971). The division

of the injected blastomere is arrested at metaphase while the uninjected one continues to divide. As a control, the injection of the cytoplasm from a prophase oocyte is unable to arrest the cell division of the injected blastomere. This cytostatic activity was called CSF. As for MPF, researchers began investigations to identify the molecular identity of CSF. Four properties have to be satisfied for a protein to be part of the CSF: (1) it is inactive in prophase-arrested oocytes, (2) it appears during meiotic maturation, (3) it is inactivated after fertilization or following the parthenogenetic activation of the oocyte and (4) its injection in a blastomere of a two cell-embryo must promote an arrest in metaphase (Masui *et al.* 1971). CSF is conserved among vertebrates and it is now well established that CSF is tightly linked to the Mos/MAPK pathway.

ii. The Mos/MAPK pathway

The first protein identified as a CSF component was Mos. Mos satisfies all the CSF criteria: it is absent in prophase, accumulates in response to progesterone, is activated at the time of GVBD and is stable until fertilization that provokes its proteasome-dependent degradation (Sagata *et al.* 1988, Watanabe *et al.* 1989, Lorca *et al.* 1991, Castro *et al.* 2001a). Moreover, the injection of Mos or of any of its targets, MEK, MAPK or $p90^{Rsk}$, under constitutively active forms, in a blastomere of a two-cell embryo arrests the injected blastomere in metaphase (Sagata *et al.* 1989, Haccard *et al.* 1993). Finally, cytoplasm from *Xenopus* MII oocytes that are depleted in Mos loses its CSF activity, *i.e*. the ability to arrest blastomeres in metaphase (Sagata *et al.* 1989). The demonstration of the physiological involvement of the Mos/MAPK pathway during the MII arrest came from the analysis of knock-out Mos mouse oocytes. In this species, oocytes without Mos proceed through a third meiotic division without arresting in metaphase II, and in some cases, keep dividing, giving raise to an abnormal embryonic body that eventually dies (Colledge *et al.* 1994, Hashimoto *et al.* 1994b, Verlhac *et al.* 1996b, Verlhac *et al.* 2000). The role of the Mos/MAPK pathway in the second meiotic arrest is well conserved among sea urchin, starfish, ascidians, nematodes, jellyfish, *Drosophila*, fishes, *Xenopus* and mouse oocytes, despite the arrest of oocytes at various steps of meiotic maturation (prophase, MI, MII, G_1) according the species (Tachibana *et al.* 1997, Dupre *et al.* 2002, Mori *et al.* 2006, Amiel *et al.* 2009, Aze *et al.* 2010). Importantly, immunodepleting $p90^{Rsk}$ or MAPK or adding U0126 is not sufficient to release *Xenopus* CSF extracts from M-phase and overexpressing Mos in MII-arrested oocytes does not prevent the activation of oocyte induced by Ca^{2+} (Bhatt *et al.* 1999, Reimann *et al.* 2002). In *Xenopus*, the Mos/MAPK pathway is therefore necessary for the establishment of the arrest in metaphase II but is not required for its maintenance. In vertebrates, since oocytes injected with Mos reach GVBD and proceed through meiosis II without arresting in MI,

this further indicates that a protein, produced during the MI-MII transition, cooperates with the Mos/MAPK pathway to establish the arrest at MII.

iii. Role of APC

Based on the ubiquitin-dependent degradation of Cyclin B that is necessary to inactivate Cdk1 in somatic cells, it was assumed that APC/C could be a primary target of CSF to maintain a high level of Cyclin B in MII. Consistently with this idea, the overexpression of proteins implicated in the SAC, such as Mad2, Bub1 and Mps1, block the Ca^{2+}-induced release of M-phase in *Xenopus* CSF extracts (Schwab *et al.* 2001, Tunquist *et al.* 2003, Grimison *et al.* 2006). However, when the same proteins are depleted from eggs, neither the inhibition of APC/C nor the maintenance of CSF arrest is affected (Abrieu *et al.* 2001, Sharp-Baker *et al.* 2001, Tunquist *et al.* 2003). This is in agreement with the fact that *Xenopus* oocytes are defective for the SAC (Shao *et al.* 2013) and CSF-arrested oocytes contain an intact metaphase plate that is not expected to trigger SAC activation (Masui *et al.* 1971). This result strongly suggests that SAC proteins are not active players contributing to the CSF activity and that the oocyte is equipped with a CSF-specific inhibitor of APC/C. In 2002, Emi1 was proposed to be this CSF-specific inhibitor (Reimann *et al.* 2002). Recombinant Emi1 is able to induce a mitotic arrest when injected into a blastomere of a two cell-embryo and its immunodepletion from CSF extracts leads to APC/C activation and Cyclin B degradation (Reimann *et al.* 2002). However, the physiological relevance of Emi1 as CSF was rapidly challenged. Emi1 is stable in prophase and both ectopic and endogenous Emi1 are highly unstable proteins during meiotic maturation (Margottin-Goguet *et al.* 2003, Tung *et al.* 2005). Moreover, the arrest in MII promoted by injected Emi1 is not released upon oocytes activation (Ohsumi *et al.* 2004).

Another intriguing issue is that the incubation of MII-arrested oocytes with CHX promotes a partial degradation of the Cyclin B and a decrease of Cdk1 activity up to 50% of its initial level, without provoking the release from the MII arrest (Thibier *et al.* 1997). While there is no overall variation in the concentration of Cyclin B during the MII arrest, a fraction of Cyclin B is however under turnover while the other one is stabilized. Hence, the CSF arrest is a dynamic process wherein the expression level of Cyclin B could be regulated by two mechanisms: APC/C and the Mos/MAPK pathway. Both actors were reconciled by the finding of Erp1/Emi2.

iv. Erp1/Emi2

Upon fertilization, the activity of the kinase CaMKII is increased by the peak of Ca^{2+} and results within less than 5 minutes in the activation of APC/C and the degradation of Cyclin B (Lorca *et al.* 1993). In contrast, the Mos/MAPK pathway remains active for at least 30 minutes following egg activation or

fertilization (Castro *et al.* 2001a). Therefore, fertilization bypasses the protection ensured by the Mos/MAPK module to trigger Cyclin B degradation through an unknown mechanism. This bypass can be achieved by the inactivation of an unknown effector of the Mos/MAPK pathway. The intermediate protein was identified in a yeast two-hybrid screen seeking proteins interacting with catalytically inactive Plx1 and was called Erp1/Emi2 (Liu *et al.* 2005a, Schmidt *et al.* 2005). Erp1/Emi2 is an inhibitor of APC^{Cdc20} that acts by direct binding (Shoji *et al.* 2006, Wu *et al.* 2007b). In CSF extracts, Erp1/Emi2 is fully degraded within 5 minutes after Ca^{2+} addition and its immunodepletion promotes the release from the metaphase arrest in the absence of Ca^{2+} (Schmidt *et al.* 2005). The mechanism responsible for its degradation was further elucidated. Following oocyte activation, Erp1/Emi2 is phosphorylated by CaMKII, which provides a Plx1 docking site necessary for its degradation by SCF_{β}^{TrCP} E3 ubiquitin ligase (Liu *et al.* 2005a, Rauh *et al.* 2005, Schmidt *et al.* 2005, Hansen *et al.* 2006). The inhibition of APC/C is then relieved and promotes the exit from MII. Altogether, these results strongly suggest that Erp1/Emi2 is the long though CSF-specific inhibitor of APC/C that is responsible for mediating the arrest in MII. Although the endogenous expression of *Xenopus* Erp1/Emi2 during the prophase arrest as well as during meiosis resumption is still under debate, Erp1/Emi2 is not present at a sufficient level in MI to prevent Cyclin B degradation since its injection at GVBD is sufficient to arrest the oocyte in MI (Ohe *et al.* 2007). Clearly, the protein is accumulated at a high level when the oocytes enter in MII (Schmidt *et al.* 2005, Ohe *et al.* 2007). Importantly, Erp1/Emi2 is required for the CSF activity of the Mos/MAPK pathway (Inoue *et al.* 2007, Nishiyama *et al.* 2007). Recombinant Erp1/Emi2 injected at GVBD loses its ability to arrest oocytes at MI in the presence of U0126 (Inoue *et al.* 2007). Moreover, injected Mos is no longer capable of arresting blastomers at M-phase when Erp1/Emi2 expression is suppressed by morpholino antisense (Inoue *et al.* 2007). The link between Erp1/Emi2 and the Mos/MAPK module is mediated by $p90^{Rsk}$ that directly phosphorylates Erp1, increasing its stability and its ability to interact and inhibit APC/C (Inoue *et al.* 2007, Nishiyama *et al.* 2007) (**Fig. 22**). Strikingly, Cdk1 also phosphorylates Erp1/Emi2 at two distinct residues, T545 and T551, decreasing Erp1/Emi2 interaction with APC/C (Wu *et al.* 2007b) . These Cdk1-dependent phosphorylations of Erp1/Emi2 are antagonized by the activity of PP2A. This process may be used by the oocyte to finely tune the level of Cyclin B accumulation to ensure that the process of fertilization and the following embryonic cell cycles are not impaired. Interestingly, the $p90^{Rsk}$-dependent phosphorylation of Erp1/Emi2 promotes the recruitment of PP2A to the protein (Wu *et al.* 2007a, Wu *et al.* 2007b). The binding of PP2A to Erp1/Emi2 then induces its dephosphorylation at T545 and T551, enhancing its binding to APC/C and thus its inhibition (**Fig. 22**). Collectively, these important findings show that the Mos/MAPK pathway promotes the arrest in MII by regulating Erp1/Emi2 through $p90^{Rsk}$-dependent phosphorylation, which promotes APC/C

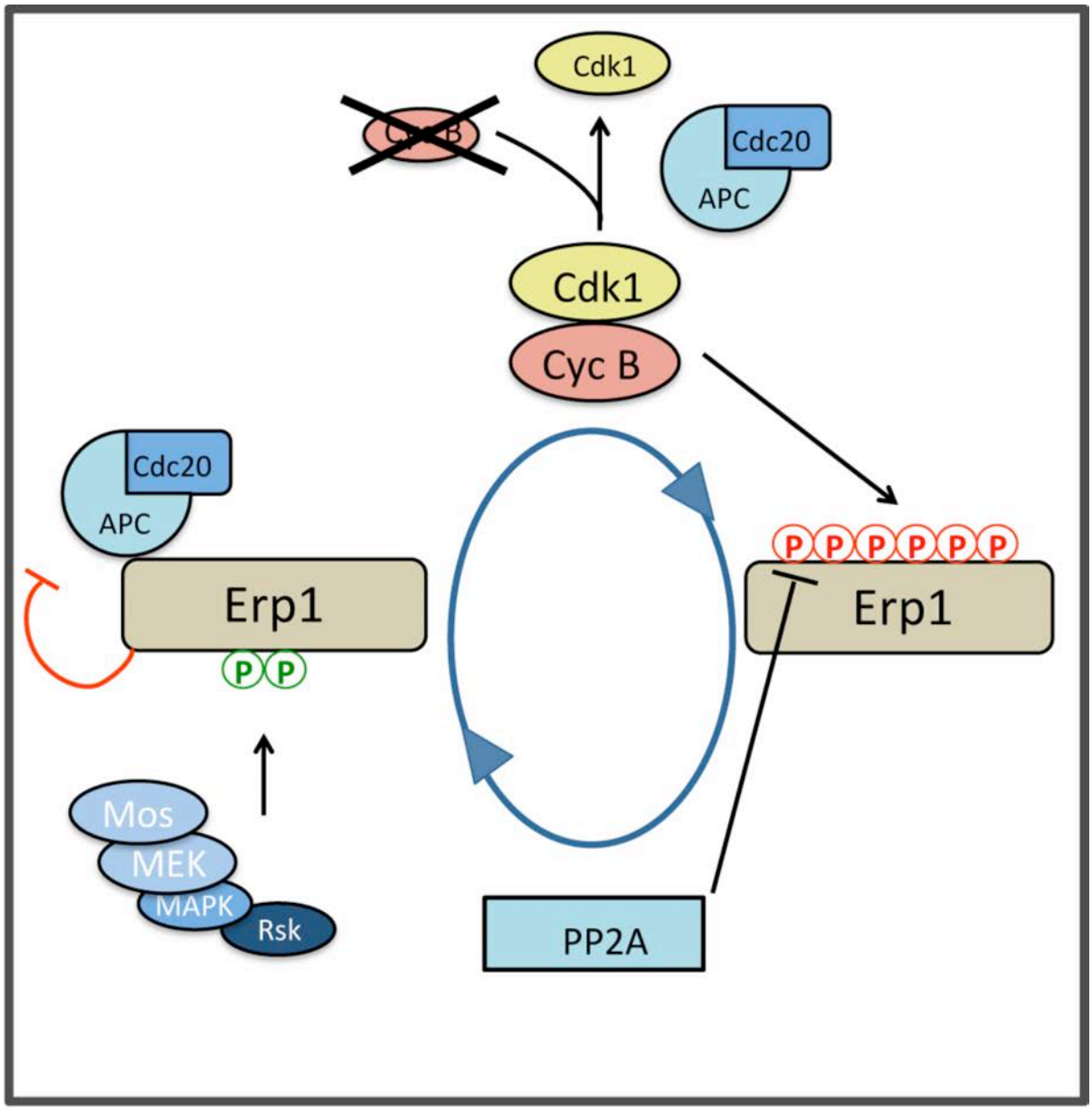

Fig. 22: the arrest in MII is a dynamic process

The Mos/MAPK/p90^{Rsk} pathway phosphorylates Erp1 and increases its stability and its ability to inhibit APC^{Cdc20}. Once CSF is established, the ability of Erp1 to bind and inhibits APC depends on the equilibrium between Cdk1 and PP2A activities. This feedback mechanism contributes to maintain a constant level of Cdk1 activity during the CSF arrest by regulating Cyclin B stability.

inhibition, the stability of Cyclin B and therefore the MII arrest. Exit from this arrest is provoked by the degradation of Erp1/Emi2 due to the CAMKII that is activated by the Ca^{2+} surge, bypassing the effects of the $p90^{Rsk}$ phosphorylation.

This long story shows that it was illusory to search for one protein as the CSF factor, since CSF is not a single factor but rather a dynamic process involving many enzymatic actors. It also addresses the question of the control of meiotic arrests in invertebrates oocytes. In these species the Mos/MAPK module is clearly involved, but obviously with other targets than Erp1/Emi2 and APC/C as their arrest occur at other phases than M-phase.

CHAPTER II:
Arpp19 and the arrest in prophase I

II – Arpp19 and the arrest in prophase I

The first part of my Ph.D. project regards the underlying mechanisms responsible for arresting the oocyte in prophase of the 1st meiotic division. This arrest is conserved in all animal species, lasts for an extraordinary long period used by the oocytes to accumulate nutrients and determinants essential for the embryonic development. This arrest is commonly assimilated to a G_2 arrest and is released by external stimuli, progesterone in *Xenopus*, which initiates a signalling pathway whose ultimate goal is to activate MPF. Remarkably, while the molecular mechanisms controlling the activation of MPF are well understood, little is known about the mechanisms ensuring for the remarkably durable prophase I arrest (2-3 years in *Xenopus* but up to 40 years in human). In a similar manner, little is known on how these mechanisms are either unlocked or circumvented by external stimuli to induce re-entry into meiotic divisions.

In all vertebrates, the maintenance of prophase arrest requires that cAMP and the activity of the cAMP-dependent protein kinase (PKA) be maintained at high levels in the oocyte. This conclusion has been supported by many studies and led to the conclusion that a phosphoprotein, a specific PKA substrate, is responsible for the prolonged prophase arrest of the oocyte. Thus, PKA plays an important role in keeping Cdk1 inactive during the prophase I arrest of meiosis in vertebrates. However, the crucial questions of the identity of the essential substrate of PKA and its molecular roles have not been solved. The study of this physiological process should lead to great advances in our knowledge of hormonal regulation, cell cycle regulation and signal transduction.

A. The endosulfine family

The endosulfine family comprises two main members encoded by different genes: the endosulfines (α and β) and the cAMP-phosphoregulated proteins including Arpp16, Arpp19 and Arpp21, base on their N-terminus end. Endosulfine was identified as an endogenous ligand of sulfonylurea receptor K^+ channels in the pancreas (Virsolvy-Vergine *et al.* 1992), whereas Arpp members were first identified in the nervous system as *in vitro* substrates for PKA (Walaas *et al.* 1983). While Arpp16 is mainly expressed in brain and is conserved in birds and mammals, Arpp19 is ubiquitously expressed in every tissue and is conserved in all eukaryotes from yeast to human (Girault *et al.* 1990). Little is known about the structure of these little proteins. ENSA behaves as an unfolded protein in solution and forms a 4 α-helixe-structure, when incubated with lipid mimetics (Boettcher *et al.* 2008). These proteins are heat stable and acid resistant and are thought to be monomeric.

a. Phosphorylation of endosulfines

Four distinct sites of phosphorylation have been identified in these proteins (**Fig. 23**). The best conserved motif, FDSGDY, is present in all the proteins of the endosulfine family and is targeted by the kinase Gwl (Gharbi-Ayachi *et al.* 2010, Mochida *et al.* 2010)(Labandera *et al.* 2015). Cdk1 targets two sites: preferentially S28 but also T99 *in vitro*, and also in *Xenopus* CSF extracts (Mochida *et al.* 2010)(Mochida 2013). The last site, S109 in *Xenopus* (S107 in human), is phosphorylated by PKA *in vitro* and in *Xenopus* extracts (Mochida *et al.* 2010)(Mochida 2013). In CSF extracts derived from oocytes arrested at metaphase II, both Gwl and Cdk1 sites of Arpp19 are phosphorylated (Mochida 2013). Following the addition of Ca^{2+}, CSF extracts exit from the CSF arrest and S67 of Arpp19 is quickly dephosphorylated in a OA-dependent manner whereas the Cdk1-dependent phosphorylation of Arpp19 remains stable for up to 30 minutes before vanishing (Mochida 2013). Interestingly, in CSF extracts, the phosphorylation of Arpp19 at S109 has never been observed neither during arrest in M-phase nor following the return in interphase. However, this phosphorylation can be induced by adding cAMP analogues (Mochida 2013).

The phosphorylation at S67 of Arpp19 by Gwl converts Arpp19 in an inhibitor of PP2A-B55δ (Gharbi-Ayachi *et al.* 2010, Mochida *et al.* 2010), and this process has been well characterized in many animal models both in mitosis and in meiosis (See chapters: I-A.e.ii.2. "Role of protein phosphatase" and I-B.d.iv.2. "Greatwall and PP2A"). On the contrary little is known on the function of Arpp19 phosphorylation by Cdk1. Recently, this function was investigated in starfish oocytes where it was shown that Cdk1 phosphorylation was promoting Arpp19 ability to inhibit PP2A independentently of Gwl (Okumura *et al.* 2014) (See chapter: I-B.d.iv.2. "Greatwall and PP2A"). Finally, it has been proposed that the S107 phosphorylation alters the secondary structure of human ENSA, since the phospho-mimetic mutant protein, S107E-ENSA, become unable to form the forth α-helixes following its incubation with lipid mimetics (Boettcher *et al.* 2008). The PKA site is very well conserved in vertebrates, while it tends to be lost in lower eukaryotes.

B. Working hypothesis

Interestingly, the injection of 50 pmol of ATP-γ-S in prophase arrested oocytes was shown to inhibit progesterone-induced meiotic maturation (Belle *et al.* 1984). The proteins phosphorylated by using ATP-γ-S as a phosphate donor are resistant to phosphatases and hence are stabilized under a phosphorylated state. It was then hypothesized that ATP-γ-S stabilizes the PKA-dependent phosphorylation of maturation phosphorylated proteins (MpP), whose dephosphorylation is required

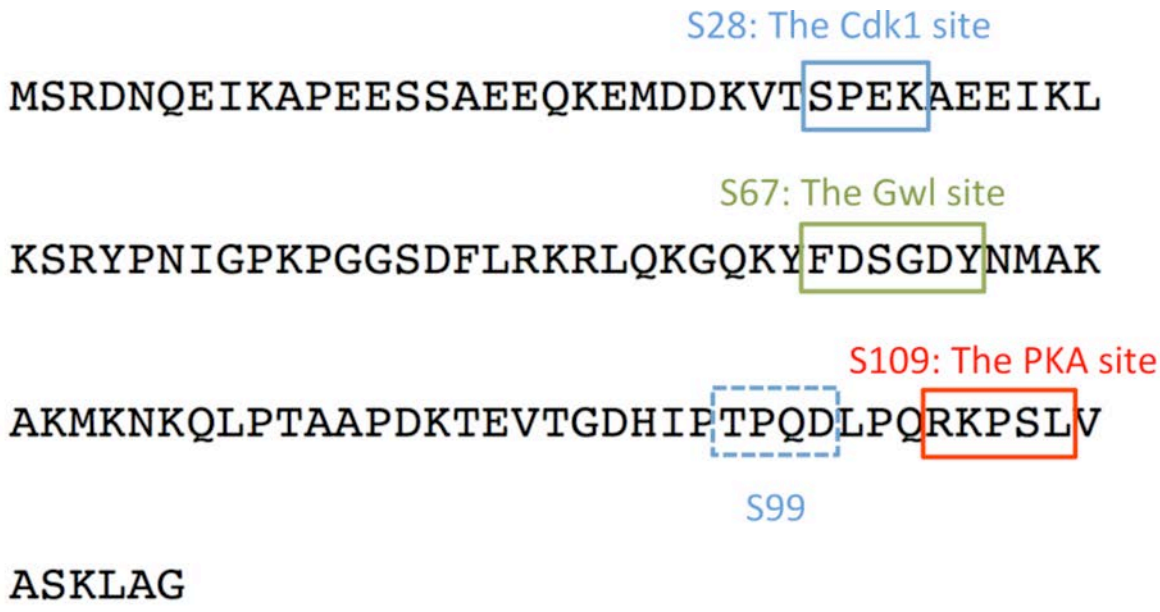

Fig. 23: The sequence of *Xenopus* Arpp19

Xenopus Arpp19 is known to be phosphorylated at four distinct sites: S28 and S99 are phosphorylated by Cdk1, S67 by Gwl and S109 by PKA. While the function of the Gwl-dependent phosphorylation of Arpp19 has been well characterized in mitosis and in meiosis, the role of the PKA and Cdk1-dependent phosphorylations of Arpp19 remains elusive.

for meiosis resumption. In order to identify these PKA substrates, oocytes were injected with PKA-C together with radiolabeled γ^{32}-ATP. Under this condition, the phosphorylation level of two proteins of 32 kDa and 20 kDa was increased compare to oocytes injected with only γ^{32}-ATP (Boyer *et al.* 1986). It was further showed that the 20 kDa protein, but not the 32kDa one, was the only one incorporating γ-S^{35}-ATP in prophase arrested oocytes (Boyer *et al.* 1986). These results suggest that the thiophosphorylation by PKA of the 20 kDa protein could be responsible for arresting oocytes in prophase following the injection of γ-S-ATP. In good agreement with this hypothesis, the addition of progesterone leads to the early dephosphorylation of the 20 kDa protein in oocytes injected with γ^{32}-ATP (Boyer *et al.* 1986). Finally, a causal relationship between these two putative PKA substrates, the 20 kDa and the 32 kDa proteins, was enlightened by injecting first γ-S-ATP and then γ^{32}-ATP. The general pattern of γ^{32}-ATP incorporation was then compared between oocytes pre-injected with ATP-γ-S or not (Boyer *et al.* 1986). While the 20 kDa protein slightly incorporated ^{32}P, the 32kDa protein, which cannot incorporate ATP-γ-S, was much more phosphorylated than in control oocytes. This experiment indicates that the irreversible phosphorylation of the 20 kDa protein increases the phosphorylation of the 32kDa protein. The 20 kDa protein could exert this function by activating a kinase or by inhibiting a phosphatase. While, the 20 kDa protein was partially purified based on its acid stability and heat resistance (Boyer *et al.* 1986), its molecular identity was never solved.

Since the biochemical properties of Arpp19 are very closed to the 20 kDa MpP and given that Arpp19 is a substrate of PKA, we then wondered whether the 20 kDa MpP could be Arpp19. At first glance, this hypothesis looks provocative, since Arpp19 is known to be a positive regulator of M-phase through its Gwl-phosphorylation at S67. How could it be responsible for a G_2 arrest while it is essential to promote M-phase? Interestingly, depending on the amount of injected protein, wild-type-Arpp19 (WT-Arpp19) is able to produce opposite effects on meiosis resumption induced by progesterone. At low concentration, WT-Arpp19 is able to stimulate Cdk1 activation. This enhancing effect of the low dose of WT-Arpp19 was proposed to be mediated by its S67 phosphorylation through the Cdk1-dependent activation of Gwl (Dupre *et al.* 2013). In contrast, injected at high concentration, WT-Arpp19 is able to abolish Cdk1 activation in response to progesterone. We therefore attempted to test the hypothesis that Arpp19 could be the 20 kDa MpP protein described about 30 years ago (Boyer *et al.* 1986). We performed the same purification protocol to isolate the 20 kDa MpP and submitted the heat and acid-stable fractions of oocytes from prophase and metaphase II to western blot with an anti-Arpp19 antibody. Arpp19 was strongly detected at 20 kDa in these fractions. From these results, we decided to investigate whether Arpp19 is the long searched substrate of PKA responsible for the prophase arrest.

C. Results: the phosphorylation of Arpp19 by the protein kinase A prevents Cdk1 activation in *Xenopus* oocytes

To analyse the role of Arpp19 phosphorylation at S109 during meiosis resumption, we generated an Arpp19 phospho-mimetic mutant by mutating the S109 into an aspartic acid (S109D-Arpp19) and a non phosphorylatable form of Arpp19 by mutating the S109 into an alanine (S109A-Arpp19).

a. Effects of S109A- and S109D-Arpp19 mutants on meiosis resumption induced by progesterone or PKI

These two mutant proteins were injected in prophase oocytes and their effects on meiosis resumption were ascertained by western blotting several markers of Cdk1 activation: Gwl, Mos, phosphorylated MAPK, Cyclin B2, Cdc27. As controls, we further used recombinant WT-Arpp19, known to either block or facilitate meiosis resumption in response to progesterone, as well as recombinant S67A-Arpp19, which cannot be phosphorylated at S67 and prevents both progesterone and PKI ability to induce meiosis resumption (Dupre *et al.* 2013). While the S109A-Arpp19 mutant had no effect on GVBD induction and Cdk1 activation in response to progesterone, the S109D-Arpp19 abolished meiosis resumption upon progesterone addition. Injected at a same concentration, WT-Arpp19 delayed GVBD induction whereas the S109A-Arpp19 mutants has little effect on Cdk1 activation following hormonal stimulation, suggesting that S109 phosphorylation mediates the inhibitory effect of WT-Arpp19. Noteworthy, S109A-Arpp19 was unable to activate Cdk1 on its own. These results indicate that the activation of Cdk1 and meiosis resumption are inhibited when the phosphorylation of Arpp19 at S109 is maintained.

b. S109D-Arpp19 blocks the initial activation of Cdk1 but not the MPF autoamplification loop

If the S109D-Arpp19 plays the role of the early substrate of PKA, the injection of this mutant is expected to block the first Cdk1 activation induced by PKI. In contrast, the same mutant should be unable to block the second step, the MPF auto-amplification, induced by the injection of either okadaic acid, S67-thio-phosphorylated-Arpp19, constitutively active K71M-Gwl or cytoplasm transfer. Indeed, these approaches launch the MPF autoamplification loop independently of PKA activity, with the exception of K71M-Gwl that is unable to induce meiosis resumption in the presence of IBMX (Dupre *et al.* 2013).

Meiosis resumption was triggered by injecting PKI in oocytes previously injected with either WT-Arpp19 or S109D-Arpp19. While Cdk1 was activated in oocytes injected with WT-Arpp19, Cdk1

activation in the presence of the S109D-Arpp19 mutant never occurs. On the other hand, injecting S109D-Arpp19 did not inhibit the MPF autoamplification triggered by okadaic acid, K71M-Gwl, S67-thio-phosphorylated-Arpp19 or cytoplasm transfer. Therefore, keeping Arpp19 phosphorylated at S109 prevents the initial activation of Cdk1 but is unable to block meiosis resumption directly induced by the MPF autoamplification loop. These results argue for Arpp19 being the substrate of PKA whose dephosphorylation is needed for the initial activation of Cdk1

c. Endogenous Arpp19 is phosphorylated by PKA in prophase and is dephosphorylated within one hour following progesterone addition

All the previous data were obtained using recombinant proteins injected into oocytes. We then addressed the question of the endogenous Arpp19 as the early substrate of PKA. If this hypothesis is correct, Arpp19 must be phosphorylated by PKA in prophase and should be dephosphorylated within one hour after progesterone addition in a CHX-independent manner. Using a specific antibody directed against the S109-phosphorylated form of Arpp19, we discovered that Arpp19 is indeed phosphorylated at S109 in prophase-arrested oocytes. We further monitored the phosphorylation statute of injected WT-Arpp19 at S109. As expected, the protein was phosphorylated at S109 in prophase. To ascertain that this S109 phosphorylation was dependent on PKA activity, oocytes were injected with WT-Arpp19 then injected with PKI. Following the injection of PKI, both endogenous Arpp19 and recombinant WT-Arpp19 were quickly and fully dephosphorylated at S109 within 30 minutes. This demonstrates that Arpp19 is phosphorylated at S109 in a PKA-dependent manner in prophase-arrested oocytes.

We then analysed the dephosphorylation at S109 of endogenous and recombinant Arpp19 in response to progesterone. Upon hormonal stimulation, both proteins were dephosphorylated within one hour independently of protein synthesis. Importantly, the S109 dephosphorylation of endogenous Arpp19 was partial, about 50% of the Arpp19 proteins remaining phosphorylated at that site. Thereafter, Arpp19 was fully rephosphorylated at S109 around GVBD. This rephosphorylation was still observed in oocytes injected with PKI, showing that a kinase distinct from PKA is responsible for the phosphorylation of Arpp19 at S109 at GVBD. Therefore, endogenous Arpp19 behaves as injected WT-Arpp19 and fulfils the criteria for being the prophase-specific PKA substrate.

d. The extent of WT-Arpp19 dephosphorylation at S109 depends on progesterone concentration

In *Xenopus*, PKI as progesterone induce GVBD in a dose-dependent manner (Maller *et al.* 1977, Huchon *et al.* 1981c, Rime *et al.* 1992b). This suggests that the extent of the dephosphorylation of the PKA substrate should vary as a function of progesterone concentration. Since WT-Arpp19 is phosphorylated by PKA in prophase-arrested oocytes, more WT-Arpp19 protein is injected, higher is the level of S109 phosphorylation. This could explain why WT-Arpp19 prevents Cdk1 activation in response to progesterone in a dose-dependent manner.

To address this question, prophase oocytes were injected with either WT-Arpp19 or S109D-Arpp19 (600 ng per oocyte, a concentration of WT-Arpp19 that blocks meiosis resumption) then incubated with the usual concentration of progesterone (1 µM). This high amount of WT-Arpp19 prevented both GVBD induction and Cdk1 activation. The concentration of progesterone was then raised to 10 µM for both WT- or S109D-Arpp19 injected oocytes. Under this condition, both GVBD and Cdk1 activation were induced in oocytes injected with 600 ng of WT-Arpp19. In contrast, 10 µM progesterone remained unable to promote meiosis resumption in S109D-Arpp19 injected oocytes. In agreement with these results, WT-Arpp19 was weakly dephosphorylated at S109 in response to 1 µM progesterone whereas the extent of its dephosphorylation at S109 was significantly increased in response to 10 µM progesterone. Therefore, the extent of S109 dephosphorylation is regulated by the concentration of progesterone and accounts for the negative effect of Arpp19 on meiosis resumption. Noteworthy, this provides the first clue to understand the variability of the oocyte response to the concentrations of progesterone depending on the females.

D. Discussion

Altogether, our results demonstrate that Arpp19 phosphorylation at S109 by PKA is essential for maintaining the oocyte arrested in prophase. This represents a breakthrough that opens new avenues for understanding the control of meiotic divisions, and more broadly cell cycle regulation.

a. Arpp19, a tool to investigate the role of PKA during meiosis resumption

A signalling pathway is often considered as a chain of "events" (including the expression/degradation of a protein, the activation/inhibition of an enzyme, the change of localization or of interactors for a particular factor) that are ordered to provoke a final effect. The order of these events in a signalling pathway can be deciphered by artificially manipulating them and by performing epistatic analysis. For example, two events (1 and 2) positively regulate the signalling pathway: the "event 1" is activated while the "event 2" inhibited, if the final effect is still observed it means that the "event 1" acts downstream the "event 2". Reversely, if the final effect is blocked, it can be concluded that the "event 1" is upstream the "event 2".

The signalling pathway induced by progesterone and leading to GVBD could be schematized as a linear pathway: the hormonal stimulation promotes the decrease of cAMP levels that downregulates PKA, which in turn leads to protein synthesis and the generation of a starter amount of active Cdk1. This starter amount of active Cdk1 then triggers the MPF autoamplification loop, which catalyses the conversion of the pre-MPF into active MPF and is necessary for GVBD (the final event that is easy visualized). However, this basic view of meiosis resumption as a linear pathway has been challenged these last years. Firstly, Cyclin B and Mos synthesis can trigger independently of each other the activation of Cdk1, suggesting that at least two parallel pathways are acting redundantly to induce GVBD. Secondly, the injection of Cyclin B induces GVBD when protein synthesis is blocked with CHX (Huchon *et al.* 1993b), but loses this ability when PKA activity is maintained at a high level, with IBMX (Rime *et al.* 1992a). These results strongly suggest that PKA locks meiosis resumption by acting both upstream and downstream protein synthesis. As a consequence, any treatments used to keep high PKA activity will systematically lock the signalling pathway at two different steps, making impossible to perform a correct epistasis analysis (See chapter: I-B.d.iv. "the two-steps mechanism of Cdk1 activation") (**Fig. 24**).

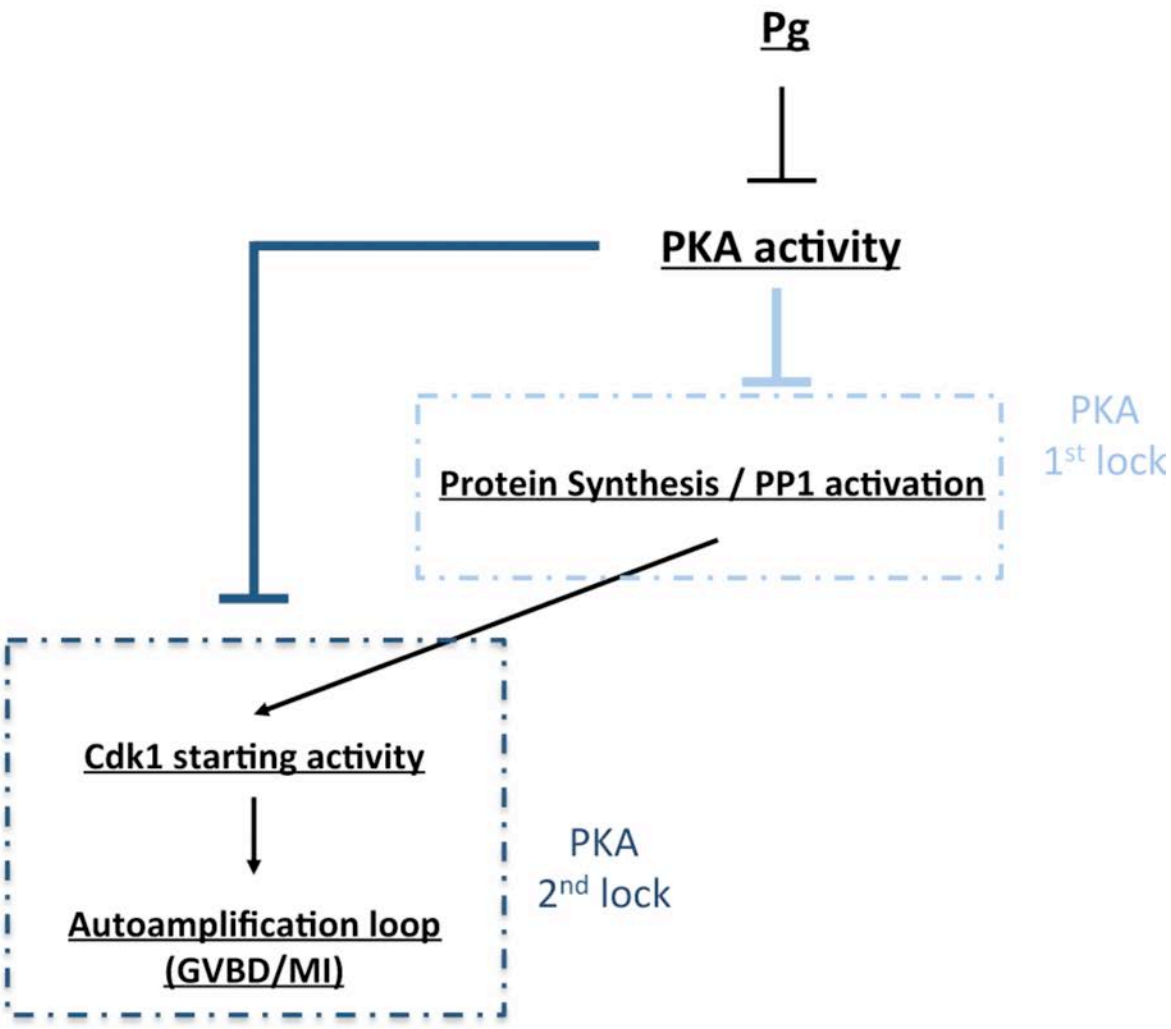

Fig. 24: A high level of PKA activity locks meiosis resumption at two different levels

PKA activity locks the G_2/M transition at two levels in *Xenopus* oocytes: the 1st lock occurs upstream proteins synthesis and PP1 activation while the 2nd lock is actived downstream protein synthesis.

Our work demonstrates that the phospho-mimetic mutant of Arpp19, the S109D, blocks the ability of progesterone and PKI in triggering GVBD whereas it has no effect on GVBD induction following the injection of okadaic acid or the cytoplasmic transfer from a MII-arrested oocyte. Altogether, these results strongly argue for Arpp19 being the PKA substrate which blocks the early signalling pathway triggered by progesterone. Therefore, the injection of PKI together with the S109D-Arpp19 mutant could be used as a tool to maintain the early PKA lock and suppress the second PKA lock that lays downstream protein synthesis (**Fig. 25**). Under this condition, the injection of Cyclin B might be able to induce GVBD. This approach would be a convenient way to test the involvement of events occurring during the window separating S109 dephosphorylation of Arpp19 from protein synthesis: any event occurring within this window should be able to induce GVBD in oocytes previously injected with both PKI and S109D-Arpp19, but not in oocytes incubated with CHX.

It is well established that PKA locks Cdk1 activation at several steps, but once Cdk1 is completely activated in the automplification loop it become insensible to the PKA lock. Is MPF automplification loop dominant over PKA activity only because it is occurring downstream PKA locks? or it is because once it is able to actively downregulate or keep inhibited PKA activity? To answer that question Arpp19 phosphorylation at S109 can be further used as a tool to track the activity of PKA during the MPF automplification loop. Arpp19 is rephosphorylated at S109 at GVBD, independently of PKA activity and in a protein synthesis dependent manner. To address this question the injection of okadaic acid will lunch the automaplification loop indepdendently of protein syntheisis. The phosphorylation status of WT-Arpp19 injected at GVBD and of endogenous Arpp19 at S109 will indicated if the MPF automplification loop inhibits PKA activity independent of hormonal stimulation.

b. The underlying mechanisms controlled by S109 phosphorylated Arpp19 during the prophase arrest

i. The active form of Arpp19: S109p-Arpp19 versus S109-Arpp19

Does Arpp19 phosphorylated at S109 block a step required for Cdk1 activation (hypothesis 1)? Does the phosphorylation at S109 exclude Arpp19 from playing its role in the early steps of meiosis resumption (hypothesis 2)? In other words, does S109 phosphorylation of Arpp19 render the protein inhibitory towards Cdk1 activation (hypothesis 1), or does it prevent Arpp19 to play a positive role in meiosis resumption (hypothesis 2)? Are both mechanisms involved in controlling meiosis resumption? (**Fig. 26**). These issues are difficult to answer because endogenous Arpp19 is expressed at the protein level and very stable all over meiotic maturation and its expression cannot be

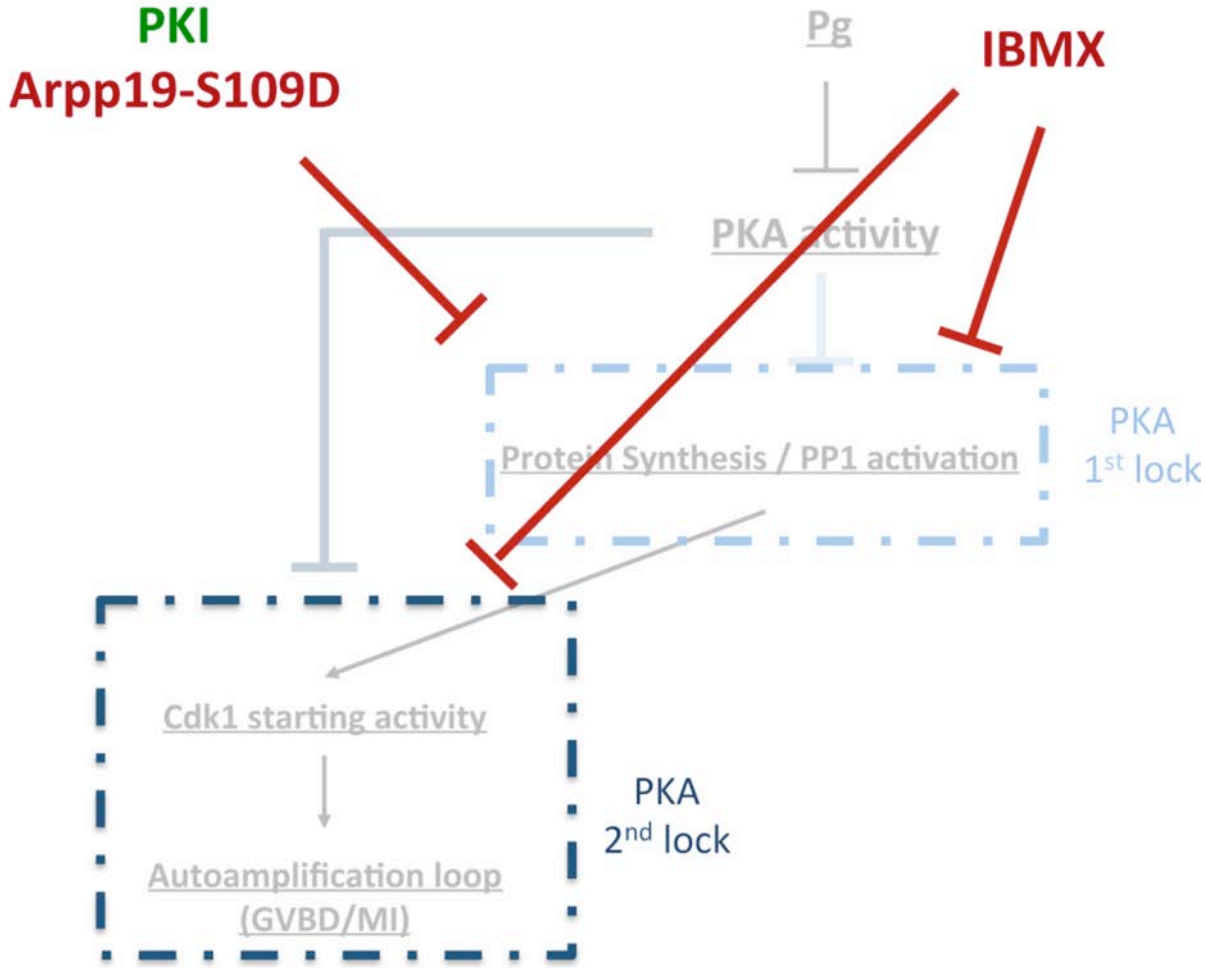

Fig. 25: IBMX versus PKI and S109D-Arpp19

The incubation of oocytes with IBMX blocks meiosis resumption by acting upstream and downstream protein synthesis. The injection of PKI together with S109D-Arpp19-S109D should inhibit Cdk1 activation by acting only during the early step in response to progesterone, *i.e.* upstream protein synthesis.

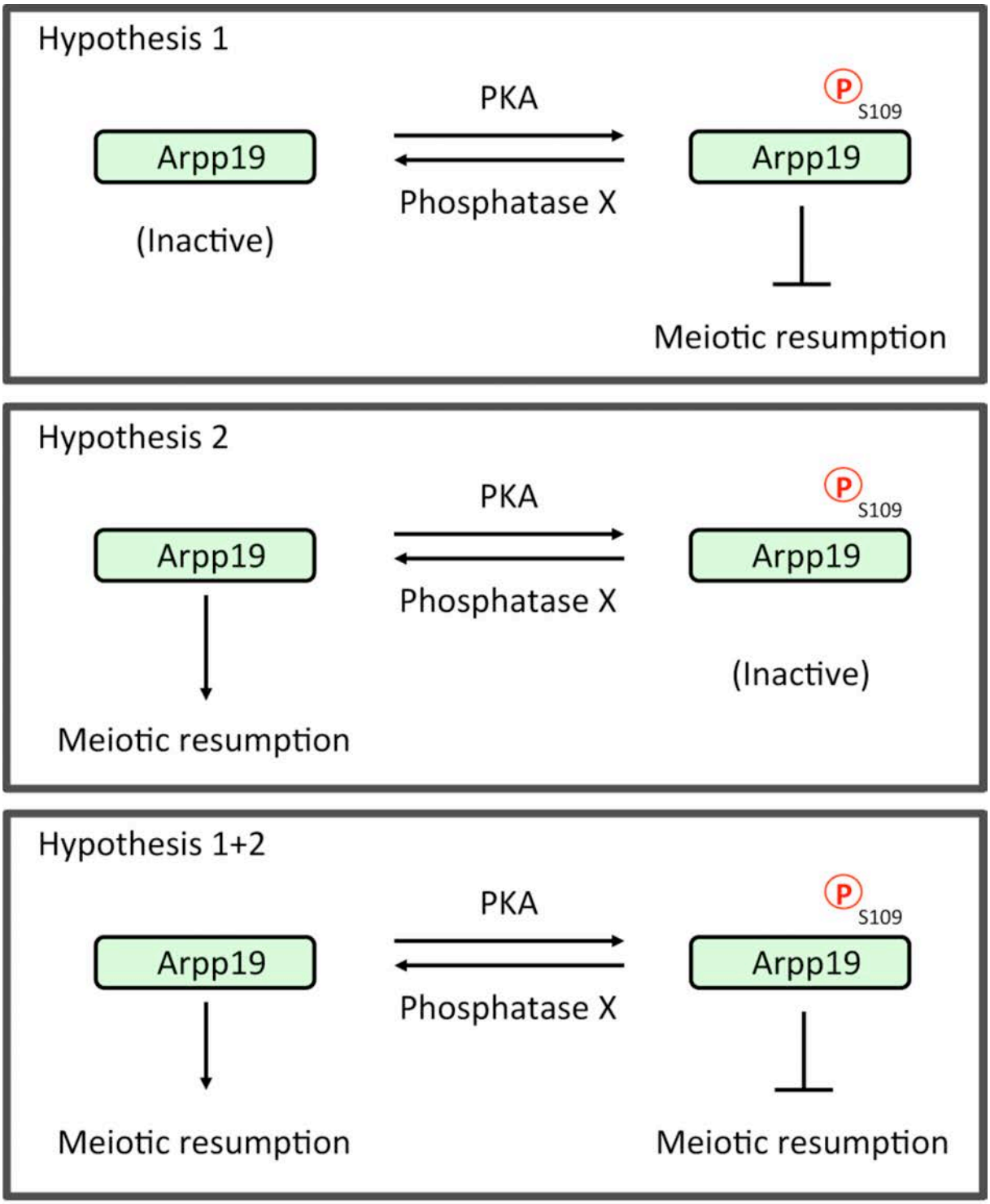

Fig. 26: S109p-Arpp19 versus S109-Arpp19

A major issue that needs to be addressed regarding the role of Arpp19 during meiosis is to determien which form of Arpp19 is the active one in the oocyte. Does S109p-Arpp19 actively block a step required for resumption (Hypothesis 1)? Does the phosphorylation at S109 inhibit Arpp19 from playing its role as an inducer of meiosis resumption (Hypothesis 2)? Are both hypothesis valid (Hypothesis 1+2) ?.

suppressed with antisense oligonucleotides. Secondly, as mentioned above, PKA activity blocks at two distinct steps during meiotic resumption.

S109D-Arpp19 blocks Cdk1 activation in response to progesterone or following PKI injection. This result supports the idea that the S109 phosphorylation blocks a step required within the initial activation of Cdk1 (Hypothesis 1). Under this condition, S109 of endogenous Arpp19 should be dephosphorylated, unless S109D-Arpp19 locks its own phosphatase (see Chapter II.D.b.iv.1. "Arpp19, PP1 and the prophase arrest"). However, since we do not know the phosphorylation status of endogenous Arpp19, the second hypothesis cannot be formally excluded. In addition, the injection of S109A-Arpp19 is unable on its own to induce meiosis resumption independently of Progesterone. This result demonstrates that the sole dephosphorylation of Arpp19 at S109 is not sufficient to activate Cdk1 and thus exclude the hypothesis II. However, since PKA still locks Cdk1 activation downstream protein synthesis, thus could explain the inability of S109A-Arpp19 to induce maturation. In order to maintain only the early PKA lock, PKI with S109D-Arpp19 should be injected. These oocytes, which are still blocked in prophase, can be further injected with increasing concentration of S109A-Arpp19 to see whether GVBD induction is restored. This experiment was performed and injectioning S109A-Arrp19 cannot bypass the inhibitory effect exerted by S109D-Arpp19. These results strongly support that S109D-Arpp19 is actively blocking a step required for Cdk1 activation. Hence, the phosphorylated form of Arpp19 at S109 is the active form of the protein inhibiting meiosis resumption and the dephosphorylated form of Arpp19 at S109 probably does not regulate Cdk1 activation (Hypothesis 1).

ii. Interplay between the phosphorylations of Arpp19 at S109 and at S67

In *Xenopus* oocyte, the Gwl-dependent phosphorylation of Arpp19 at S67 converts this protein into a potent inhibitor of PP2A-B55δ, a key event required for the activation of the MPF autoamplification loop (Dupre *et al.* 2013). Overexpressing a mutant of Arpp19 that cannot be phosphorylated at S67, the S67A-Arpp19 mutant, blocks Cdk1 activation in response to progesterone or following PKI injection (Dupre *et al.* 2013). This mutant acts as a dominant negative over Gwl and was shown to prevent the S67 phosphorylation of endogenous Arpp19; indicating that, PP2A-B55δ is unlikely to be inhibited under this condition. On the other hand, injecting a form of Arpp19, previously *in vitro* thiophosphorylated by Gwl at S67, S67*-Arpp19, binds efficiently PP2A and activates Cdk1 independently of progesterone and of PKA downregulation (Dupre *et al.* 2013). Altogether, these results clearly demonstrate that the inhibition of PP2A-B55δ by S67-phosphorylated Arpp19 is both necessary and sufficient for meiosis resumption. Therefore, S109-phosphorylated Arpp19 could impede Cdk1 activation by preventing its own ability to inhibit PP2A. This negative effect of S109p-

Arpp19 can be exerted in two ways: the inhibition of Arpp19 phosphorylation at the Gwl-site or blocking the binding to PP2A (**Fig. 27**).

S67*-Arpp19 as well as K71M-Gwl are able to trigger meiosis resumption in the presence of S109D-Arpp19 (Dupre *et al.* 2013). This result indicates that the constitutive phosphorylation of Arpp19 at S67 is dominant "in trans" over the negative effect induced by S109D. Moreover, injected S109D-Arpp19 is phosphorylated at S67 at GVBD following the injection of okadaic acid or cytoplasm transfer (Dupre *et al.* 2013). This experiment suggests that S109 phosphorylation of Arpp19 does not affect Gwl ability to phosphorylate Arpp19 at S67; at least when Gwl is fully activated. Since Gwl and S67-phosphorylated Arpp19 are involved within the MPF autoamplification loop, these results are in good agreement with the dominance of the MPF autoamplification loop over the PKA lock. However, okadaic acid and cytoplasmic transfer do not recapitulate the two-step mechanism required for Cdk1 activation in response to progesterone. Therefore, we cannot rule out the possibility that the S109 phosphorylation of Arpp19 decreases the affinity of Gwl for Arpp19 and/or delays the kinetic of S67-phosphorylation in a physiological situation, such as in response to progesterone.

To gain some insights regarding this mechanism, the kinetic of S67 phosphorylation could be *in vitro* analysed using active wild type Gwl, immunoprecipitated from MII-arrested oocytes (K71M-Gwl cannot be used due to its altered substrate specificity), in the presence of either WT-Arpp19, S109D-Arpp19 or S109A-Arpp19. Moreover, it would be possible to perform a time course experiment in progesterone-stimulated oocytes previously injected with a physiological concentration of either WT-Arpp19 or S109A-Arpp19 to follow S67 phosphorylation. In these oocytes, recombinant Arpp19 will compete with endogenous Arpp19 for Gwl phosphorylation. Therefore, the level S67 phosphorylation of endogenous and recombinant protein can be analyse in the same oocytes. If the S109 phosphorylation somehow delays the S67 phosphorylation, the S109A-Arpp19, but not WT-Arpp19, will be phosphorylated at S67 in advance compared to endogenous Arpp19. Altogether, these experiments will give us a hint regarding a direct relation between S109 and S67 phosphorylation status exists (**Fig. 27**).

Recently, it was shown in starfish oocytes that the Cdk1-dependent phosphorylation of Arpp19 at S69 during meiotic maturation was able to inhibit PP2A (Okumura *et al.* 2014). Importantly, this inhibition of PP2A-B55δ is independent of the Gwl dependent phosphorylation of Arpp19 (Okumura *et al.* 2014). *Xenopus* Arpp19 possesses two potential sites of phosphorylation for Cdk1, S28 and S99, with S28 being more efficiently phosphorylated *in vitro* by Cdk1 (Mochida *et al.* 2010). Whether Cdk1 phosphorylates Arpp19 and has a function in Cdk1 autoamplification during *Xenopus* meiotic maturation is unknown. If so, it will be important to determine whether a crosstalk exits between the S109 and S28 phosphorylations of Arpp19 by performing similar experiments than the ones described for Gwl.

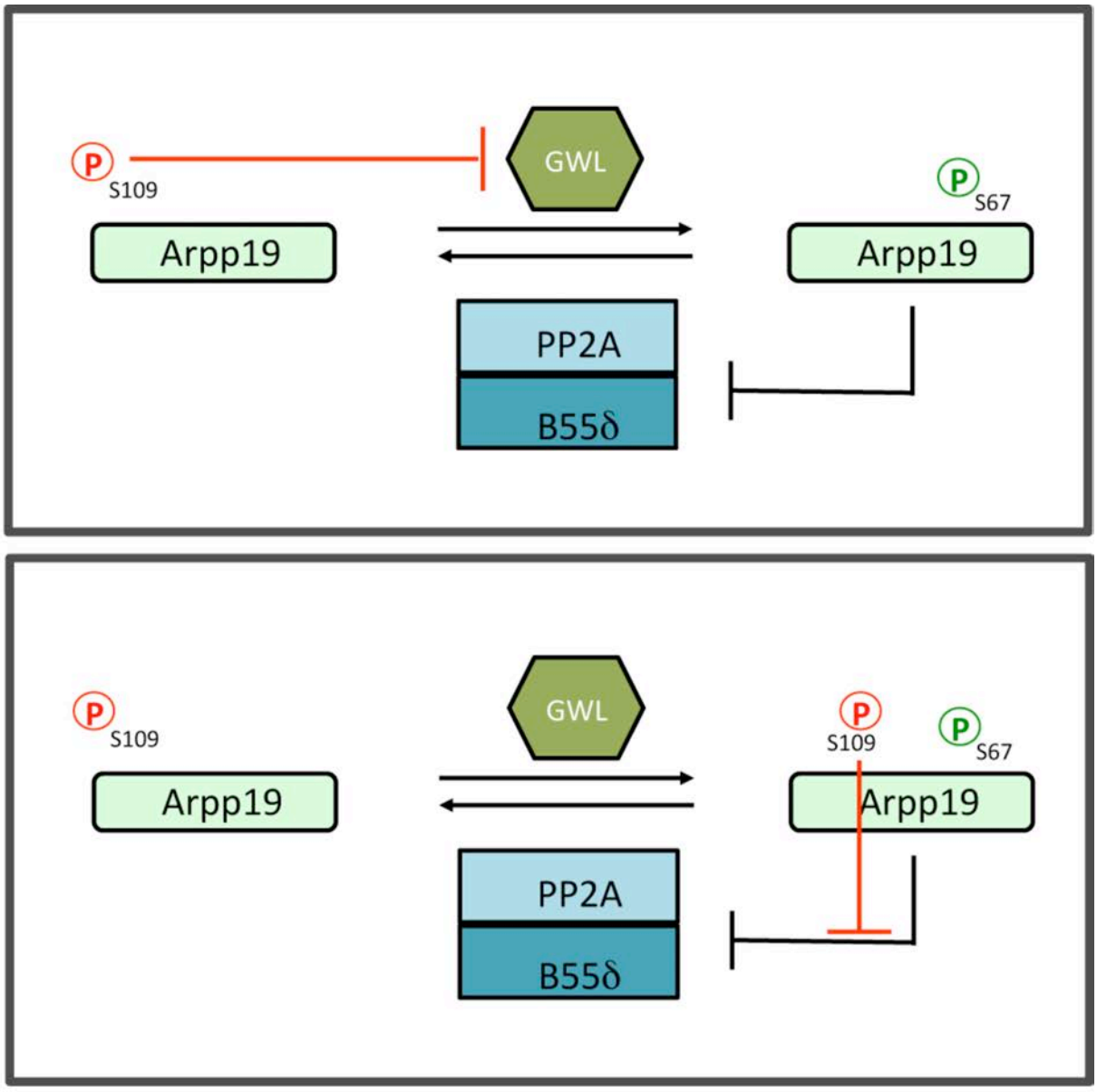

Fig. 27: Relation between Arpp19 phosphorylations at S109 and S67

The phosphorylation of Arpp19 at S109 could inhibit *in cis* its ability to inhibit PP2A in two ways. The S109-dependent phosphorylation of Arpp19 could decrease Gwl affinity for Arpp19, therefore decreasing the kinetic of Arpp19 phosphorylation at S67. On the other hand, S109 phosphorylation could decrease the affinity of Arpp19 for PP2A once Arpp19 has been phosphorylated at S67 by Gwl.

A second hypothesis is that S109 phosphorylation of Arpp19 prevents directly the ability of Arpp19 to interact with PP2A (**Fig. 27**). To answer this question, Cdk1 activation as well as the binding of Arpp19 to PP2A should be analysed in oocytes injected with either S67*-Arpp19, S109D-S67*-Arpp19 or S109A-S67*- Arpp19. If S109D-S67*-Arpp19 is unable to activate Cdk1 and to bind PP2A, the phosphorylation of Arpp19 at S109 certainly blocks the functionality of the Gwl-site *in cis* (within the same protein) (**Fig. 27**).

Finally, it will be possible to measure PP2A phosphatase activity *in vitro* in the presence of a different single and double Arpp19 mutants, as it is described in Mochida (2013). If S109D-S67*-Arpp19 produces a weaker inhibition of PP2A compared to S67*-Arpp19, it will suggest that the phosphorylation of the PKA-site could attenuate the ability of Arpp19 in inhibiting PP2A in *vitro*.

Noteworthy, Arpp19 dephosphorylation at S109 occurs much earlier than the phosphorylation at S67 by Gwl. Moreover, while the S67 phosphorylation of endogenous Arpp19 relies on both protein synthesis and Cdk1 activation, the S109 dephosphorylation of Arpp19 is independent of these two events. It would be then surprising that in the oocyte, the PKA-dependent phosphorylation of Arpp19 can directly regulate its own Gwl-dependent phosphorylation or the binding to PP2A, since these events are temporarily distinct (several hours of gap) and under distinct regulations.

iii. PKA, ArpP19 and protein synthesis

In the absence of nutrient, yeast cell enter in quiescence state (G_0) by downregulating PKA activity and activating Rim15 (Gwl homolog). Rim1 then phosphorylates Igo1 and Igo2 (Arpp19 homologues) that inhibit the action of factors implicated in mRNA deccaping (Luo *et al.* 2011). In this system, Arpp19 homologues can positively control mRNA stability and therefore protein synthesis.

Since protein synthesis is a required step for Cdk1 activation in *Xenopus* oocytes and occurs downstream PKA inactivation, the phosphorylated form of Arpp19 at S109 may inhibit meiosis resumption in response to progesterone or following PKI injection by repressing the translation of specific proteins. A good marker of protein synthesis is the Cyclin B1 whose accumulation occurs in response to progesterone or PKI injection in a Cdk1-independent manner (Frank-Vaillant *et al.* 1999). In all our experiments, the accumulation of Cyclin B1 was never prevented in response to progesterone or following PKI injection in oocytes previously injected with S109D-Arpp19 (unpublished results). Therefore, PKA inhibition of protein synthesis is unlikely to be mediated through the phosphorylation of Arpp19 at S109 (**Fig. 28**).

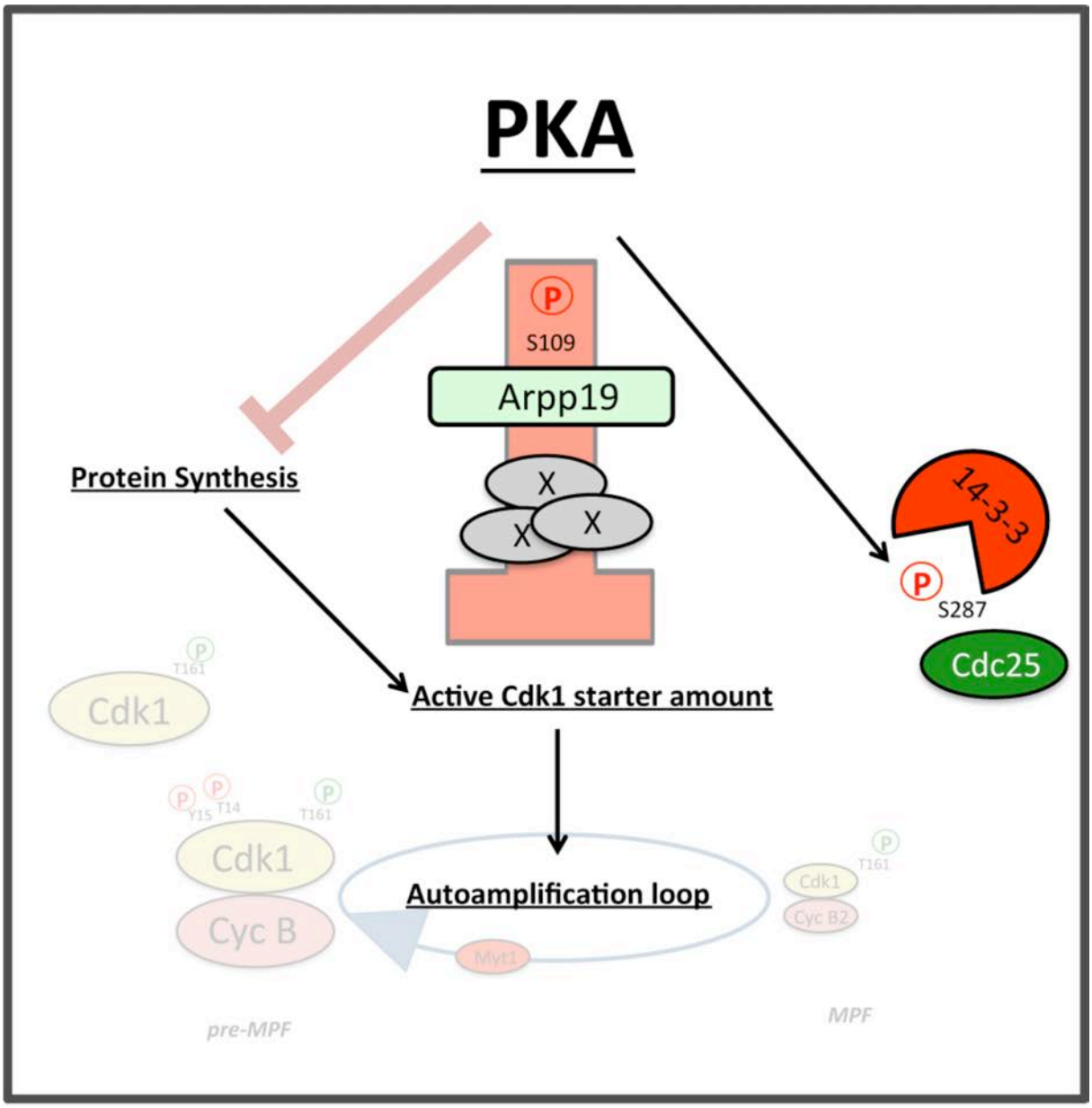

Fig. 28: Three ways for blocking meiotic maturation by PKA

PKA inhibits meiosis resumption within the early signalling pathway induced by progesterone by inhibiting protein synthesis and by phosphorylating Arpp19 at S109. Either way may be sufficient to block meiosis resumption. In addition, PKA also phosphorylates Cdc25 at S287 leading to its sequestration within the cytoplasm. However, since the dephosphorylation of Cdc25 occurs late during meiosis resumption and relies on Cdk1 activation, this event is unlikely to be involved in PKA negative effects exerted over meiosis resumption.

iv. Arpp19, an inhibitor of PP1 and PP2A phosphatases

1. Arpp19, PP1 and the prophase arrest

The investigation of PP1 activity during meiotic maturation in *Xenopus* oocytes is difficult because of several reasons. Three different catalytic subunits of PP1 (PP1C α, β and γ) are present in *Xenopus* oocytes and their regulation remains elusive during meiotic maturation (McCoy *et al.* 2013). Moreover, there is no specific chemical-inhibitors discriminating between PP1 and PP2A phosphatases. For example, OA is known to inhibit both PP2A and PP1 activity, having lower IC_{50} for PP2A. Finally, there is no identified specific substrate for PP1 in oocytes beside Cdc25, which is dephosphorylated at S287 by PP1. However, S287 dephosphorylation of Cdc25 requires the prior activation of Cdk1, in order to release Cdc25 from 14-3-3 binding, an event that is required for its dephosphorylation in mitosis (Margolis *et al.* 2006b). Therefore, Cdc25 is not a good marker to investigate PP1 activation during meiotic resumption *in ovo*.

In 1981, the injection of a specific inhibitor of PP1, the inhibitor-1 (I-1) was shsown to inhibit meiosis resumption induced by progesterone or following PKI injection (Huchon *et al.* 1981b). However, I-1 can no longer impede Cdk1 activation when injected 3 hours after the addition of progesterone and is unable to block meiosis resumption triggered by cytoplasmic transfer (Huchon *et al.* 1981b). These results suggest that the activation of PP1 is required downstream PKA inhibition as an early step to activate Cdk1. Interestingly, the 20kDa MpP, whose dephosphorylation is required for meiotic maturation, was hypothesized to be an inhibitor of protein PP1 (Boyer *et al.* 1986). Since we believe that this 20 kDa MpP corresponds to Arpp19 (Dupre *et al.* 2014), it will be interesting to investigate whether endogenous S109p-Arpp19 and S109D-Arpp19 act as an inhibitor of PP1.

The identity of the phosphatase removing phosphates incorporated at S109 of Arpp19 is unknown. It is possible to speculate that S109p-Arpp19 could be at the same time an inhibitor and a substrate of PP1, as already described for S67p-Arpp19 and PP2A inhibition (Williams *et al.* 2014). Since Arpp19 is partially dephosphorylated at S109 following progesterone addition and is rephosphorylated at S109 at GVBD, this suggests that PP1 activation could be only transitory during meiosis resumption. According to that view, S109D-Arpp19 or Arpp19 thiophosphorylated by PKA *in vitro* (S109*-Arpp19), which cannot be dephosphorylated at S109, should act as a pseudo-substrates for PP1, exerting a dominant negative effect (**Fig. 29**). If this hypothesis is correct, the co-injection of S109D-Arpp19 together with PKI is expected to block the S109 dephosphorylation of endogenous Arpp19, showing that S109D-Arpp19 blocks the phosphatase activity responsible for its own dephosphorylation. To prove that this phosphatase is PP1, injecting PKI together with okadaic acid is expected to slow down S109 dephosphorylation of WT-Arpp19 compared to PKI-injected oocytes. Furthermore, a specific inhibitor of PP1 can be injected such as I-1, previously *in vitro* thiophosphorylated at T35 by PKA to

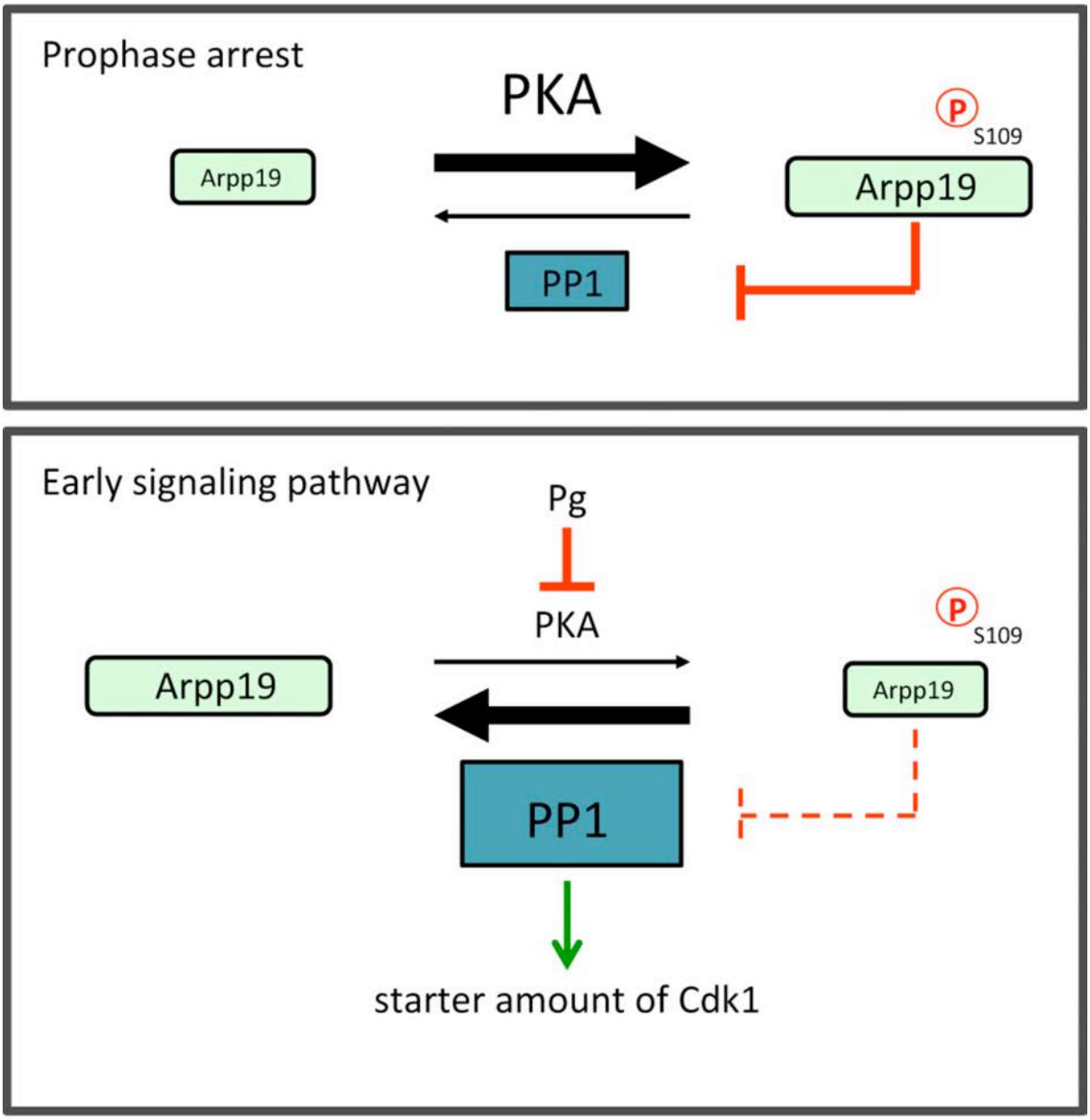

Fig. 29: Is S109p-Arpp19 an inhibitor of PP1 ?

In prophase-arrest oocytes, the high activity of PKA maintains Arpp19 phosphorylation at S109. Arpp19 could then act as a substrate and/or an inhibitor of PP1. The downregulation of PKA by blocking the renewal of S109p, Arpp19 would no longer prevent PP1 activation in response to progesterone.

maintain its inhibitory ability toward PP1 (Huchon *et al.* 1981b). Finally, if S109D-Arpp19 acts as an inhibitor of PP1, it should behave like I-1 and must lose its inhibitory effect over meiosis resumption when injected 3 hours after progesterone addition (Huchon *et al.* 1981b).

An *in ovo* approach to monitor endogenous PP1 activation could be set up using a fragment of PP1 that comprises the T320 residue (Lewis *et al.* 2013). This residue is known to be phosphorylated by Cdk1, leading to PP1 inhibition, and to be dephosphorylated by PP1 itself, at the time of its activation (Dohadwala *et al.* 1994, Wu *et al.* 2009). This peptide is efficiently *in vitro* phosphorylated by Cdk1 (Lewis *et al.* 2013) and could be injected in oocytes to monitor the activity of PP1 during meiotic maturation. If PP1 is activated in response to progesterone or following PKI injection, this peptide is expected to be dephosphorylated before the activation of Cdk1 and in oocytes injected with $p21^{Cip1}$. For all these conditions, it will be therefore interesting to determine whether the prior injection of S109D-Arpp19 blocks its dephosphorylation. Designing an *in vitro* dephosphorylation assay to monitor PP1 activity can further complement these *in ovo* experiments. The peptide could be *in vitro* phosphorylated by Cdk1 then added to immunopurified PP1 from oocytes collected at different times. The same assay could be performed in the presence of WT-Arpp19 or S109D-Arpp19 to determine whether these proteins can modulate PP1 activity.

In summary, the regulation of PP1 by S109-phosphorylated Arpp19 and the possibility of a feedback loop between these both players certainly deserves investigation. It leaves still open the question of the crucial downstream targets of PP1 that lead to Cdk1 activation. In Boyer *et al.* (1986), it was shown that the constitutive phosphorylation of the 20 kDa was increasing the phosphorylation of the 32 kDa protein. Therefore, we can speculated that S109p-Arrp19 (the 20 kDa protein) could block PP1 which activation is required to dephosphorylated the still not identified protein of 32 kDa.

2. Role of Arpp19 rephosphorylation at S109 at GVBD

Another important issue that must be addressed regards the role of Arpp19 rephosphorylation at S109 at the time of GVBD. Interestingly, okadaic acid triggers meiosis resumption in the presence of CHX (Rime *et al.* 1990) but S67*-Arpp19 cannot induce Cdk1 activation in the absence of protein synthesis (Dupre *et al.* 2013). Therefore, S67*-Arpp19 does not phenocopy okadaic acid although both of them were supposed to target the same enzyme, PP2A. A major difference between okadaic acid and S67*-Arpp19 is that the former inhibits PP1 and all PP2A holoenzymes whereas the latter is only an inhibitor of PP2A-B55δ. This observation suggests that the inhibition of another phosphatase in addition to PP2A-B55δ is necessary for GVBD and for the MPF autoamplification loop.

In mouse oocytes, the injection of blocking antibodies against PP1 is able to stimulate GVBD in roscovitine-treated oocytes (Swain *et al.* 2003). Moreover, in this model, Gwl is dispensable until MI

suggesting that the inhibition of PP2A-B55δ is not necessary for GVBD occurrence during meiosis resumption (Adhikari *et al.* 2014). From these results, it has been proposed that during meiosis resumption in mice PP1, and not PP2A-B55δ, is the phosphatase counteracting Cdk1 phosphorylation during meiosis resumption in mice and its inhibition allow the nuclear breakdown (Adhikari *et al.* 2013). During mitosis, it is well established that Cdk1 phosphorylates PP1 at T320 efficiently inhibiting its activity (Dohadwala *et al.* 1994). However, this pathway can be activated only downstream the first activation of Cdk1. According to our hypothesis, Arpp19 phosphorylated at S109 can behave as a PP1 inhibitor. Actually, we have shown that Arpp19 is rephosphorylated at S109 independently of PKA activity at GVBD time, but in a protein synthesis-dependent manner. The re-phosphorylation at S109 could be important to inhibit PP1 and to induce GVBD (**Fig. 30**). To investigate that hypothesis, it will be important to monitor the phosphorylation status at S109 of endogenous and recombinant Arpp19 protein, in CHX-treated oocytes injected with S67*-Arpp19. If S67*-Arpp19 is not phosphorylated at S109 at GVDB equivalent time, it could explain the inability of that protein to induce GVBD in CHX-treated oocytes: the CHX impairs Arpp19 re-phosphorylation at S109 blocking its ability to inhibit PP1. If this model is true, the ability to mature in CHX-treated oocytes injected with S67*-Arpp19 should be rescued by injecting S109D-Arpp19, or I-1. Moreover, it will be important to identify the pathway responsible for Arpp19 re-phosphorylation at S109, knowing that this pathway required protein synthesis. The sequence surrounding the S109 residue corresponds to a minimal consensus site for $p90^{Rsk}$ phosphorylation (RxxS). Recombinant Mos indirectly activates MAPK and $p90^{Rsk}$ independently of protein synthesis. In CHX-treated oocytes, $p90^{Rsk}$ cannot be activated in response to progesterone because of the inhibition of Mos synthesis. If $p90^{Rsk}$ is the kinase responsible for the Arpp19 rephosphorylation at S109, the injection of Mos would therefore restore the phosphorylation at S109 as well as the inhibition of PP1. Notably, Cdk1 activation is restored in CHX-treated oocytes when S67*-Arpp19 is injected together with Mos.

Importantly, this model is based on the hypothesis that S67*-Arpp19 is dephosphorylated at S109 in CHX treated oocytes. However, the activity of PKA in this experiment should be able to maintain S109 phosphorylation as progesterone was not added, unless if injection of S67*-Arpp19 is not able to downregulated the ability of PKA to phosphorylate Arpp19 at S109.

3. A new model for meiosis resumption

Based on those ideas, the following model for controlling meiosis resumption can be proposed (**Fig. 31**). The high activity of PKA maintains the phosphorylation of Arpp19 at S109. S109p-Arpp19 then

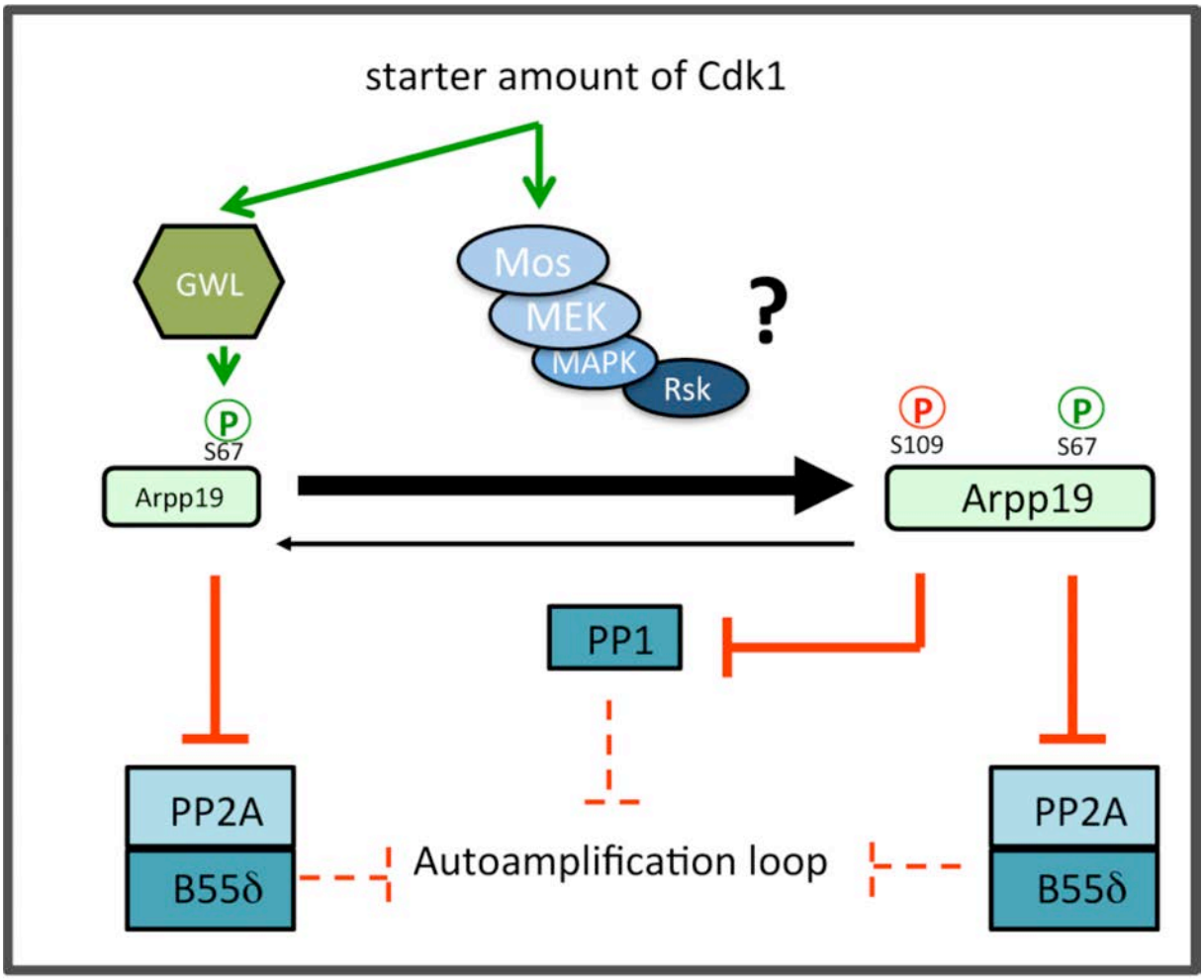

Fig. 30: S109p-Arpp19 a role around GVBD time?

Arpp19 is rephosphorylated around the time of GVBD in a PKA-independent manner and maybe under the control of $p90^{Rsk}$. This rephosphorylation of Arpp19 at S109 would convert Arpp19 in a potent inhibitor of PP1 at GVBD, an event necessary for meiotic maturation. Arpp19 is therefore an inhibitor of PP1 and PP2A phosphatases whom selectivity is conferred by its phosphorylation status at S109 or/And S67.

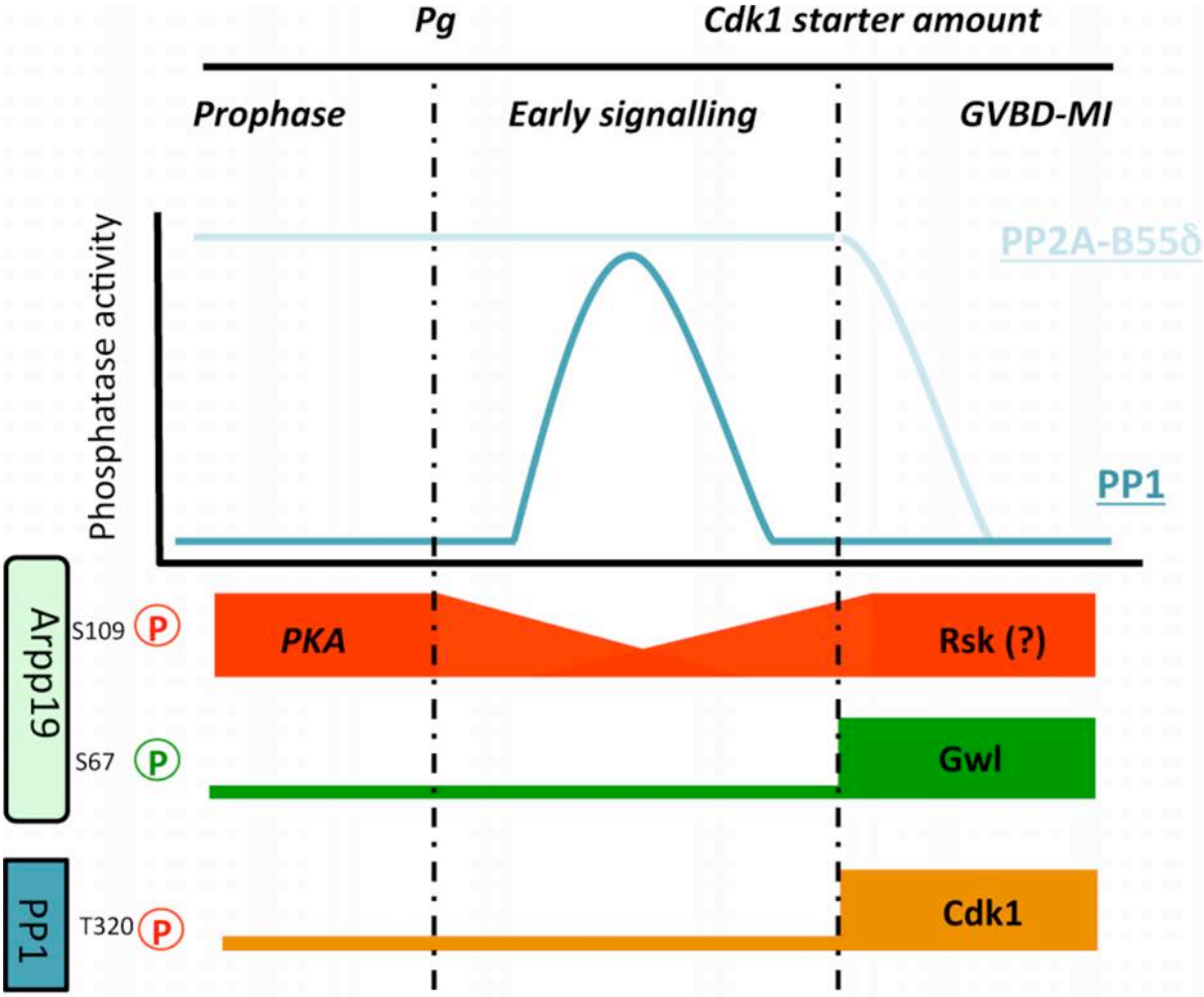

Fig. 31: Roles of protein phosphatase during meiosis resumption

In prophase-arrested oocytes Arpp19 is phosphorylated at S109, PP1 is inhibited and PP2A is active. Following progesterone addition, S109 is partially dephosphorylated within one hour leading to a transient activation of PP1, while PP2A remains active. Around GVBD time, Arpp19 is rephosphorylated at S109, independently of PKA, and at S67 by Gwl. This leads to the concomitant inhibition of PP2A and PP1 necessary for the full activation of Cdk1 and for meiosis resumption.

inhibits PP1 by being a substrate in excess (**Fig. 29**). Upon progesterone addition, the activity of PKA drops and a fraction of Arpp19 is dephosphorylated at S109. As a consequence, PP1 is activated (**Fig. 29**). The identity of its targets and their links with Cdk1 activation are still pending. Just before GVBD, the $p90^{Rsk}$-dependent phosphorylation of Arpp19 at S109 as well as the initial activation of Cdk1, which phosphorylates PP1 at T320, allows for the full inhibition of PP1. Concomitantly, the Gwl-dependent phosphorylation of Arpp19 at S67 directly inhibits PP2A-B55δ. This double phosphorylation of Arpp19 at both S109 and S67 provokes therefore the inhibition of both PP1 and PP2A-B55δ in order to fully activate Cdk1 through the MPF autoamplification loop. The oocytes then can undergo GVBD. Accordingly, Arpp19 acts as an inhibitor and a substrate of phosphatases whose selectivity is controlled by its phosphorylation status at S109 and S67, as already described for S67 and PP2A inhibition (Williams *et al.* 2014) (**Fig. 29-30**).

4. Role of Arpp19 rephosphorylation at S109 during MI-MII transition

Even though the rephosphorylation of Arpp19 at S109 would not be necessary for inhibiting PP1 at GVBD, this protein could play a role during the MI-MII transition.

In mitosis, the activity of PP1 increases in anaphase (Wu *et al.* 2009) and targets multiple substrates that coordinate processes such as chromosomes decondensation (Vagnarelli *et al.* 2011) and nuclear envelope reassembly (Steen *et al.* 2000). These events are not occurring during the MI-MII transition (Huchon *et al.* 1981a, Gerhart *et al.* 1984, Gard 1992, Furuno *et al.* 1994), suggesting that PP1 is inhibited during this phase. However, the activity of Cdk1 decreases during Anaphase I, as probably the level of the inhibitory phosphorylation at T320 of PP1. Whether the residual activity is sufficient to keep PP1 inhibited is unknown. We can propose that S109p-Arpp19, presumably maintained under its phosphorylated status by the Mos/MAPK cascade, could help inhibiting PP1 during that transition (**Fig. 32**). When the Mos/MAPK activity is inhibited with morpholino against Mos or U0126, oocytes exit from meiosis I and do not enter into meiosis II but they are driven in a pseudo interphasic state accompanied with the reformation of nuclei and DNA replication (Furuno *et al.* 1994, Gross *et al.* 2000, Dupre *et al.* 2002). It will be interesting to see if the injection of S109D-Arpp19 at GVBD is able to block nuclei reformation and, therefore, DNA replication in oocytes maturing without the Mos/MAPK pathway.

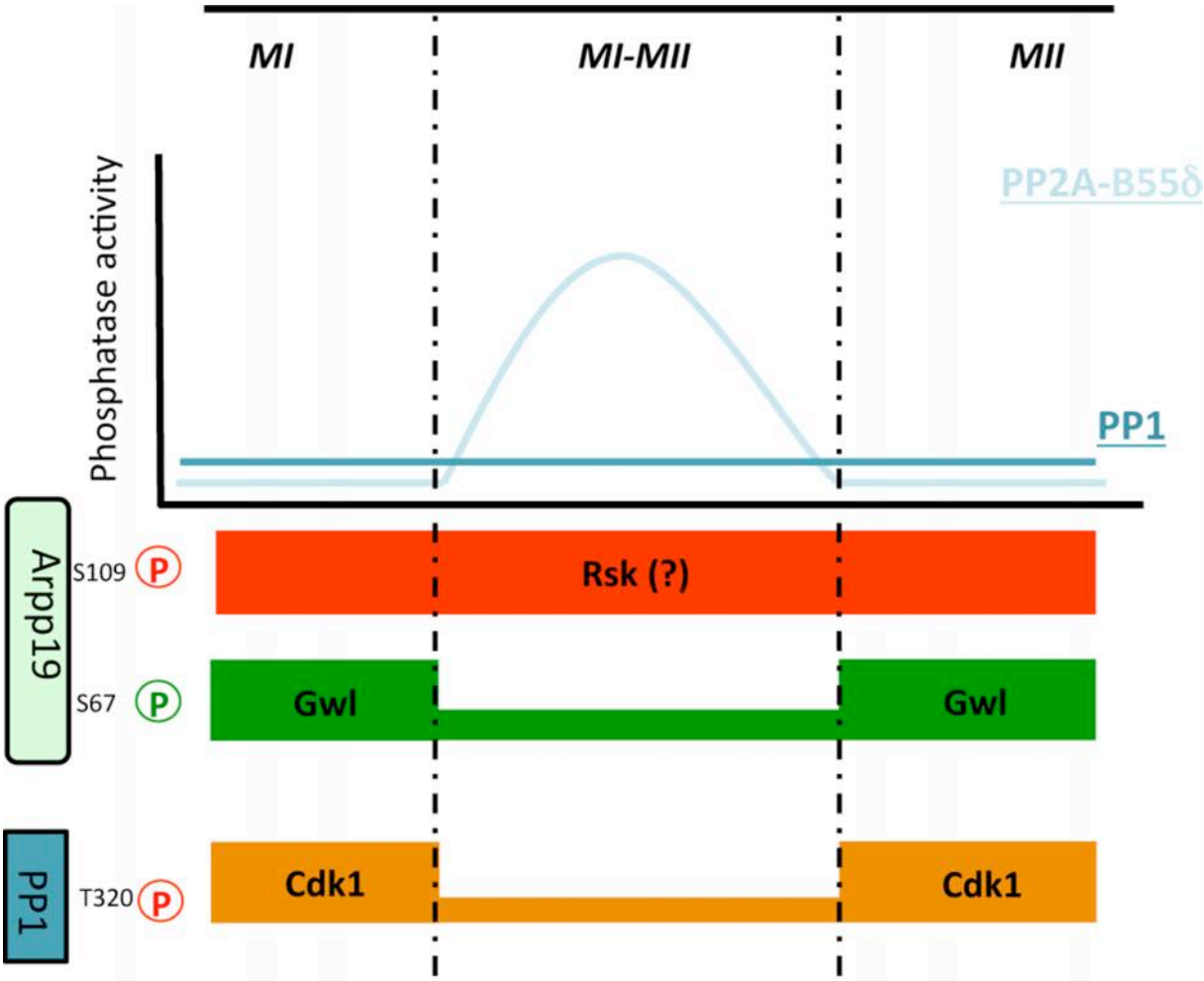

Fig. 32: Roles of PP1 and PP2A during the MI-MII transition

At GVBD, both PP2A and PP1 are inhibited. During the MI-MII transition, Cdk1 is partially inactivated, the activity of Gwl decreases and Arpp19 is dephosphorylated at S67 leading to PP2A reactivation. In addition, the partial inactivation of Cdk1 decreases the level of PP1 inhibitory phosphorylation at T320. However, PP1 could be maintained inhibited during the M-M transition because Arpp19 remains phosphorylated at S109 by $p90^{Rsk}$. The inhibition of PP1 activity then impairs chromosomes decondensation and nuclear reformation.

v. *The phosphorylation at S109 controls the localization of Arpp19: the 14.3.3 hypothesis*

The localization of the actors regulating Cdk1 activation in the oocyte could affect meiosis resumption, even though enucleated *Xenopus* oocytes are able to resume meiosis (Masui *et al.* 1971). This assumption comes from experiments showing that the activation of Cdk1 proceed differently in the absence of the nucleus, Cdk1 being slowly activated and reaching a plateau indicative of a defect within the MPF autoamplification loop (Iwashita *et al.* 1998).

It will be interesting to study the localization of endogenous Arpp19 in prophase as well as in oocytes collected 30 minutes after PKI injection to determine whether Arpp19 is present or not in the nucleus. Since Arpp19 is totally dephosphorylated at S109 upon PKI injection at that time, this would suggest whether Arpp19 localization can be regulated by its PKA-dependent phosphorylation. Then, the localization of S109A-Arpp19 is expected to be abnormal in prophase oocytes while S109D-Arpp19 localization should not be altered following PKI injection.

In somatic cell lines, PKA is known to regulate Cdc25 localization by phosphorylating S287, which blocks its nuclear import by promoting its interaction with 14-3-3 proteins. Interestingly, the S109 of Arpp19 lays within a minimal consensus site for the binding of 14-3-3 ($R_{106}xxpS_{109}$). The R106 residue within S109D-Arpp19 could be mutated into an alanine in order to prevent the interaction between Arpp19 and 14.3.3, without affecting S109 phosphorylation. If this mutation abolishes the ability of the S109D in inhibiting Cdk1 activation, this will suggest that the inhibitory effect of Arpp19 conferred by its phosphorylation at S109 is probably mediated by its binding to 14-3-3. Then, the localization of S109D-R106A-Arpp19 should be similar to S109A-Arpp19 in prophase-arrested oocytes. The interaction between Arpp19 and 14-3-3 could be further studied by performing GST pull-down experiments and confirmed *in vivo* by immunoprecipitating endogenous Arpp19 from prophase-arrested oocytes injected or not with PKI. This model would explain how Arpp19 is regulated in prophase by its phosphorylation at S109, without however answering the question of its early function in the induction of Cdk1 activation.

vi. *Finding Arpp19 interactome*

The identification of Arpp19 interactors would provide precious clues on the mechanisms regulated by S109-phosphorylated Arpp19. The immunoprecipitation of endogenous Arpp19 or GST pull down experiments of recombinant Arpp19 proteins can be performed from prophase-arrested oocytes or at a given time following either progesterone addition or PKI injection. These experiments could be analysed using a candidate driven approach (by western blot) or in an unbiased manner using mass

spectrometry. Another possibility is to fractionate extracts from prophase oocytes, injected or not with PKI, using size-excluding chromatography (Gel Filtration). This biochemical approach will ascertain whether Arpp19 is eluted with other proteins and will give a good estimation about the molecular weight of the complexes containing Arpp19. The pre-incubation of extracts with Arpp19 antibodies can be used for identifying Arpp19 interacting protein: the antibodies will bind Arpp19 complex increasing its molecular weight and modifying the elution profile of both Arpp19 and its interactors.

c. S109p-Arpp19 function during the mitotic cell cycle

The role of S67p-Arpp19 as an inhibitor of PP2A-B55δ is well conserved between the meiotic division and the mitotic cell cycle. However, nothing is known regarding the function of S109 phosphorylation during the somatic cell cycle.

i. Regulation of the G_2/M transition by PKA

PKA activity fluctuates in synchronized mammalian cells being high in interphase and low in M-phase (Burger *et al.* 1972). Moreover, keeping a high level of cAMP blocks the G_2/M transition and this inhibition can be reversed by overexpressing PKI (Stambrook *et al.* 1976, Lamb *et al.* 1991, Kurokawa *et al.* 1998). If Arpp19 is the substrate of PKA mediating these effects, the mutant S109D-Arpp19 should phenocopy the inhibition of M-phase entry caused by high cAMP levels. In the presence of S109D-Arpp19, G_2/M arrest should be resistant to PKI overexpression. These experiments would show that the PKA-dependent phosphorylation of Arpp19 at S109 is sufficient for arresting the cell cycle. If cells, in which endogenous Arpp19 is knocked down and substituted with S109A-Arpp19 (expressed at a physiological level), are treated with high cAMP level they should not arrest at the G_2/M transition. This will prove that S109 phosphorylation is necessary for mediating the negative effect of high PKA activity on the G2/M transition. However, PKA activity could control other actors than Arpp19 involved in the machinery of the cell cycle such as Cdc25 and Wee1.

ii. Checkpoint recovery

In somatic cells, Cdc25 phosphorylation at S287 is mainly controlled by Chk1 and Chk2, which are activated in response to DNA damages. Since PKA and the Chk kinases share a similar minimum consensus site of phosphorylation (R/KxxS/T), it will be interesting to determine whether Arpp19 can be further phosphorylated by Chk1 and Chk2 and involved in a checkpoint-related mechanism during the G2/M transition. Checkpoint arrest and recovery are easily and well studied in *Xenopus* cycling

extracts supplied with biotin-labelled double-stranded oligonucleotides (DS-oligo). These DS-oligos promote the activation of ATM and ATR that in turn activate Chk1 and Chk2 leading to the arrest of the cell cycle at the G_2/M transition (Peng *et al.* 2010). Under this condition, PP2A activity is required to inactivate Plx1, a kinase necessary for the checkpoint recovery (Clemenson *et al.* 2009, Peng *et al.* 2011, Wang *et al.* 2015). Recently, Gwl was also found to be important for the checkpoint recovery that starts 60 to 90 minutes after the removal of DS-oligos (Peng *et al.* 2011). The checkpoint recovery decreases the phosphorylation level of Chk1 and allows the extracts to enter into M-phase. The addition of Gwl facilitates Chk1 dephosphorylation and M-phase entry whereas its depletion postpones these events (Peng *et al.* 2010). Interestingly, during checkpoint recovery, Plx1 was found to interact directly and to phosphorylate Gwl, leading to its activation (Peng *et al.* 2011). Moreover, Gwl can facilitate Plx1 activation in an indirect manner (Peng *et al.* 2011). Possibly, the hypothetical phosphorylation of Arpp19 at S109 by Chk1 can be important to keep Arpp19 inactive, meaning unable to bind PP2A and to inhibit its activity. This would prevent the activation of a pathway that otherwise would inhibit PP2A, contribute to Plk1 activation and thus reinforce/provoke the checkpoint recovery (**Fig. 33**). Moreover, this situation would be critical for somatic cells as inhibiting PP2A-B55δ is sufficient to promote M-phase entry. The idea that Gwl function during the checkpoint recovery depends on its ability to phosphorylate Arpp19 at S67 deserves to be investigated. *Xenopus* cycling extracts would therefore be a suitable approach to examine the involvement of Arpp19 in the checkpoint maintenance through its S109 phosphorylation, and in the checkpoint recovery through its S67 phosphorylation. The inactivation of Arpp19 (as an inhibitor of PP2A-B55δ) would confer strength and robustness to the arrest. Indeed, the arrest would be secured by a high activity of PP2A-B55δ and by the low activity of Cdk1, caused by its phosphorylation at Y15 following the inactivation of Cdc25.

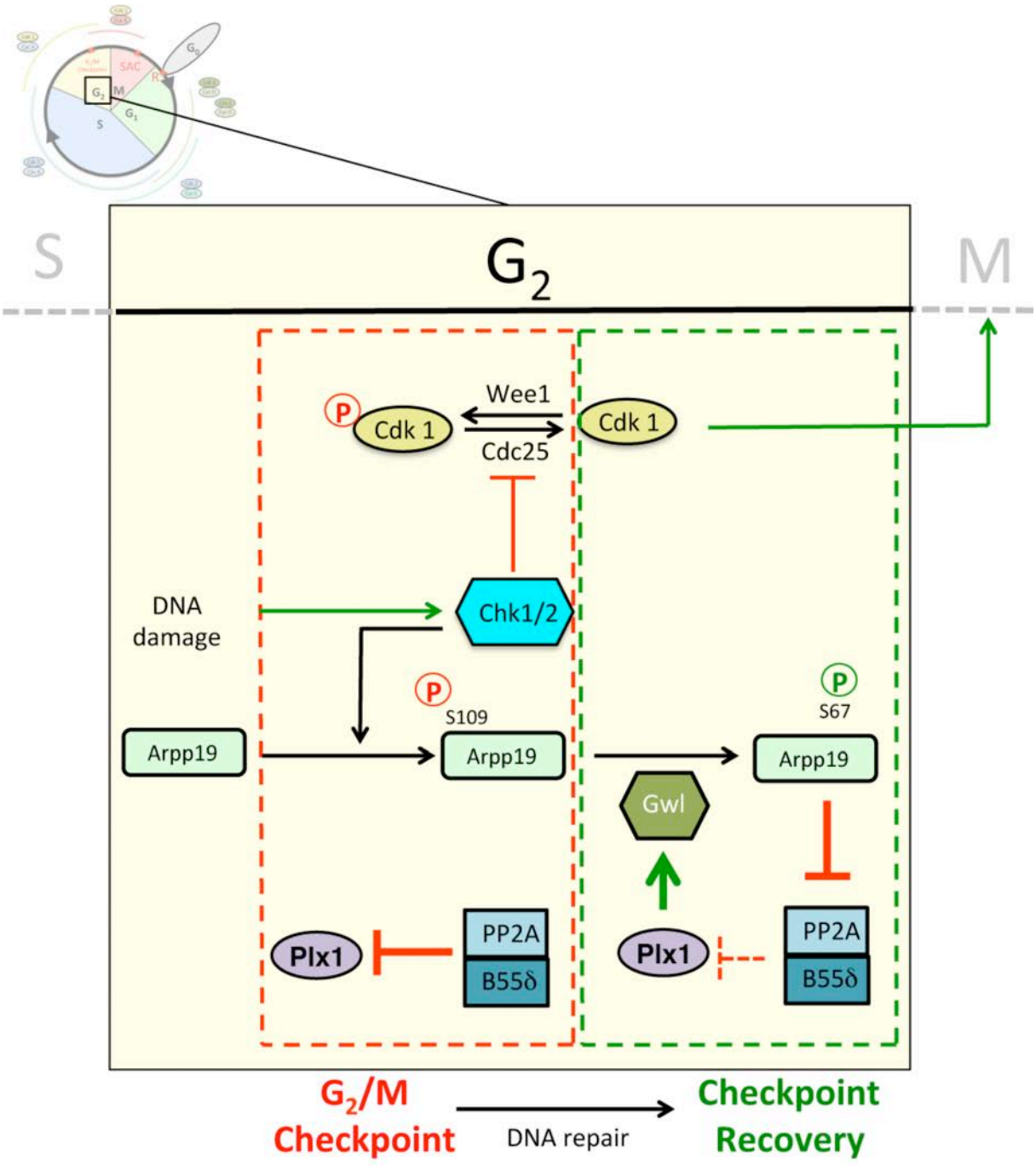

Fig. 33: Arpp19 and the G_2/M checkpoint

Following DNA damages, Chk1/2 are activated and phosphorylate Cdc25 at S287. Moreover, Chk1/2 may also phosphorylate Arpp19 at S109. Conomitantly, PP2A blocks the activation of Plx1 that is required for the checkpoint recovery together with Gwl. Once the DNA damage is repaired, Chk1/2 are inactivated and Plx1 activates Gwl. Under this condition, Arpp19 is rephosphorylated at S67 and converted into a PP2A inhibitor. The inhibition of PP2A and the reactivation of Cdc25 phosphatase then allow for the entry in M-phase.

ARTICLE I:
Phosphorylation of ARPP19 by protein kinase A prevents meiosis resumption in *Xenopus* oocytes

CHAPTER III:
The timely accumulation of Cdc6 is necessary to ensure two meiotic divisions in the absence of DNA replication in *Xenopus* oocytes

III - The timely accumulation of Cdc6 is necessary to ensure two meiotic divisions in the absence of DNA replication in *Xenopus* oocytes

Initially discovered as a major actor within the initiation of DNA replication, Cdc6 is now implicated in a plethora of mechanisms that regulate the progression of the mitotic cell cycle: re-replication control, synchronisation of S-phase with mitosis and exit from M-phase. All these functions of Cdc6 have been unravelled in different model organisms in which Cdc6 was either overexpressed or depleted. During the cell cycle, these different functions of Cdc6 are tightly regulated by its level of expression and/or its intracellular localization. An additional level of complexity is reached because these regulations have diverse effects depending on the phase of the cell cycle and on the organism. For example, the CDKs-dependent phosphorylations of Cdc6 can either stabilize the protein or promote its degradation by acting differently on APC^{Cdh1} or SCF degradation systems. Similarly, Cdc6 is fully degraded in yeast after S-phase initiation until entry into G_1 whereas in mammalian cells, Cdc6 is not completely eliminated in S- and G_2-phases and the downregulation of its function mainly involves the inhibition of its nuclear import and not its degradation.

During meiosis, little is known regarding the function and the regulation of Cdc6 beside its requirement for the competence to replicate DNA in *Xenopus* oocyte. Cdc6 is the only missing factor of the replicative machinery and its accumulation during meiotic maturation confers to the oocyte the competence to replicate DNA. During meiosis, the oocyte physiologically undergoes two consecutive M-phases without an intervening S-phase. An important issue for the oocyte is to avoid DNA replication to take place when the oocyte exits from the 1^{st} meiotic division, given the fact that it has a replication machinery ready to be used, as it should be during a regular cell cycle. Since Cdc6 is involved in the coordination of S-phase with mitosis and also regulates M-phase progression in somatic cells, we wondered whether the regulation of Cdc6 was important for the meiotic divisions.

A. Regulation of the level of Cdc6 expression

In all eukaryotes, the level of Cdc6 protein results from a tight balance between the transcription and the translation but also from the degradation of Cdc6 through different mechanisms involving ubiquitin ligases such as SCF or APC/C, whose regulation depends on the model organism and on the phase of the cell cycle.

In *S. cerevisiae* and *S. pombe*, Cdc6 accumulates in late M-phase and G_1. In these two organisms, CDK activities are able to promote Cdc6 degradation throughout the cell cycle. Therefore, at the onset of S-phase, S-Cyclins associated with CDK (Cdc28 in *cerevisiae* and Cdc2 in *pombe*) phosphorylate Cdc6

and promote its proteasome-dependent degradation by allowing SCF^{Cdc4} to interact through a F-box motif within Cdc6 (Drury *et al.* 1997, Perkins *et al.* 2001). This mechanism is important to prevent re-replication. While Cdc6 overexpression is sufficient to induce DNA re-replication in *S. pombe,* redundant mechanisms are effective to prevent re-replication in *S. cerevisiae* and deregulating Cdc6 expression is not sufficient to induce this process in this species (Nguyen *et al.* 2001). During M-phase, mitotic Cyclins bind to a site overlapping the Cdc6 F-box binding motif (Mimura *et al.* 2004). This interaction stabilizes Cdc6 by preventing its recognition by SCF^{Cdc4} and simultaneously inhibits its ability to associate with components of the pre-RCs (Mimura *et al.* 2004). In late M-phase, the degradation of mitotic Cyclins inactivates Cdk1 and releases Cdc6 that thereafter forms the pre-RCs (**Fig. 34**).

In higher eukaryotes, the stability of Cdc6 is regulated by degradation mechanisms depending on two ubiquitin ligases: APC^{Cdh1}, which recognizes the D- and KEN- boxes within Cdc6 (Petersen *et al.* 2000), and $CRL4^{Cdt2}$ recognizing a PIP-like motif in the N-terminus of the protein (Clijsters *et al.* 2014). In quiescent cells (G_0) and during early G_1, Cdc6 is ubiquitinated by APC^{Cdh1} and degraded. In late G_1, the activation of Cdk2-Cyclin E stabilizes Cdc6 and prevents its association with APC. Cdc6 is thus allowed to form the pre-RC. At the onset of S-phase, Cdc6 is phosphorylated by Cdk2-Cyclin A, and is relocalized into the cytoplasm then degraded by $CRL4^{Cdt2}$ (Petersen *et al.* 1999, Clijsters *et al.* 2014). However, it was shown that a pool of Cdc6 remains stably bound to the chromatin throughout the cell cycle (Mendez *et al.* 2000, Alexandrow *et al.* 2004), accounting for its function in mitosis. In G_2 and M-phases, soluble Cdc6 can re-accumulate, because Cdk1-Cyclin A/B inhibits $CRL4^{Cdt2}$ ubiquitination system (Rizzardi *et al.* 2015). However, Cdc6 remains in the cytoplasm due to its phosphorylation by Cdk1-Cyclin B (Clijsters *et al.* 2014) (**Fig. 35**).

B. Cdc6 and the mitotic cell cycle

a. Coordination of S-phase with mitosis

i. Role of Cdc6 in Yeast

In yeast, Cdc6-null cells fail to replicate DNA and get arrested in G1 as seen by FACS analysis and the accumulation of cells with 1N DNA content (Kelly *et al.* 1993, Piatti *et al.* 1995). This phenotype is typical of mutants with DNA replication defects. Surprisingly, after a couple of hours, the Cdc6-null cells are however able to proceed through mitosis, generating a new population of cells with 0.5N DNA content (Kelly *et al.* 1993, Piatti *et al.* 1995) (**Fig. 36**). This reduction by half of the ploidy is due to Cdc6-null cells sustaining mitosis without replicating their DNA. The ability of Cdc6 to restrain M-phase entry is independent of its function in the formation of the pre-RCs and of checkpoint activation. This conclusion was reached by using a mutant of Cdc6, Cdc6-K114E, defective for the

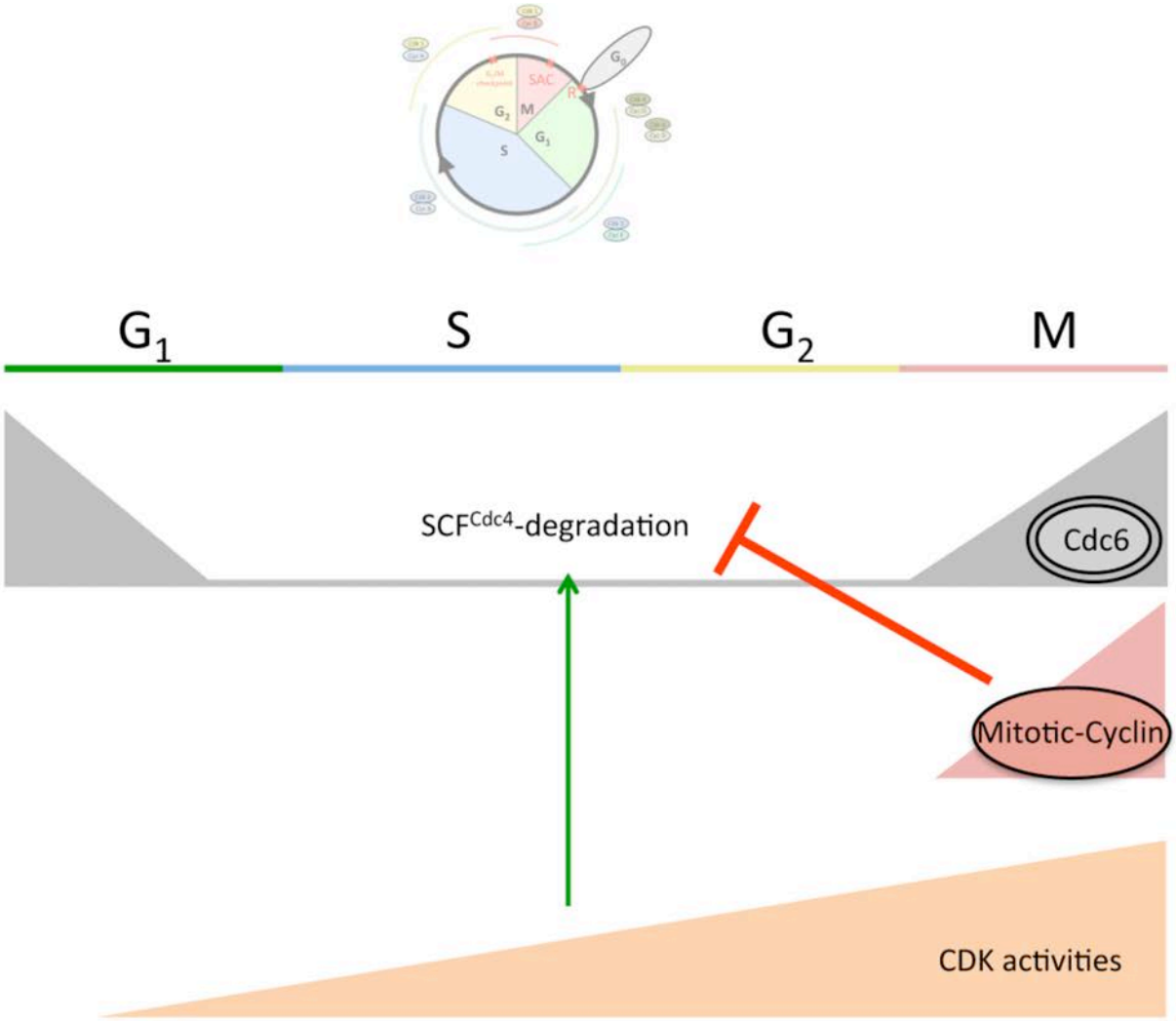

Fig. 34: Regulation of Cdc6 during mitotic cell cycle in yeast

In yeast, Cdc6 accumulation is negatively regulated by CDK activities that promotes Cdc6 degradation in a SCF^{Cdc4}-dependent manner. However, M-phase Cyclins are able to bind Cdc6. This interaction stabilizes Cdc6 and further block its ability to assemble Pre-RC. At the end of M-phase, the activation of APC/C induces the degradation of M-phase Cyclins and Cdc6 can participate to Pre-RC formation.

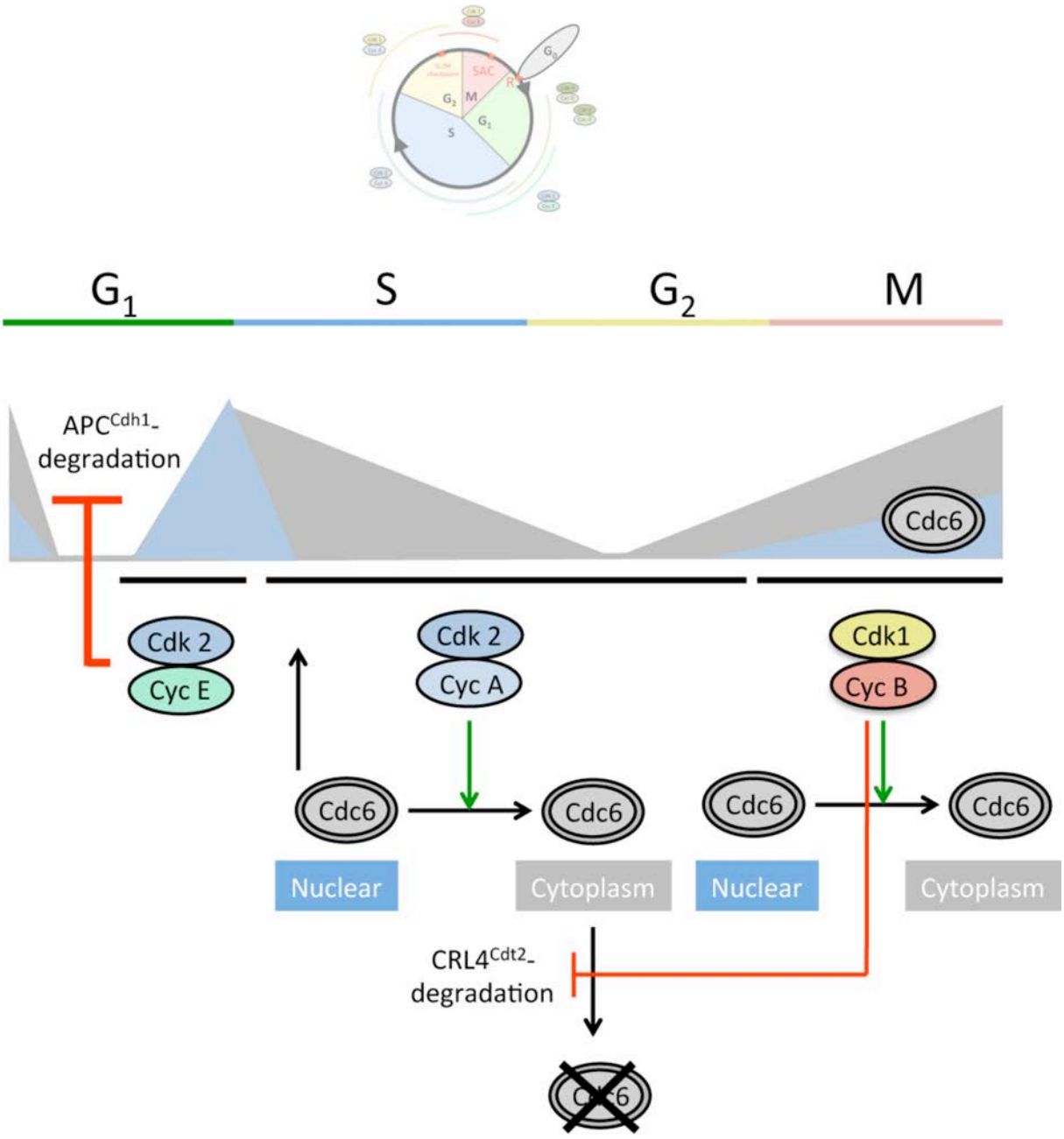

Fig. 35: Regulation of Cdc6 during mitotic cell cycle in metazoan

In higher eukaryotes, Cdc6 is present in two pools, one always bound to chromatin and the other soluble. The localization and concentration of soluble Cdc6 is tightly regulated. In G_0 and early G_1 Cdc6 is degraded in APC^{Cdh1}-dependent manner. When Cdk2-Cyclin E activates it phosphorylates and stabilizes Cdc6 that participates to the Pre-RC formation. At the G_1/S transition, Cdk2-Cyclin A licensing kinase phosphorylates Cdc6, promoting its translocation to the cytoplasm where it is degraded in $CRL4^{Cdct2}$-dependent manner. The activation of Cdk1 leads to $CRL4^{Cdct2}$ inhibition and Cdc6 can accumulated in the cytoplasm.

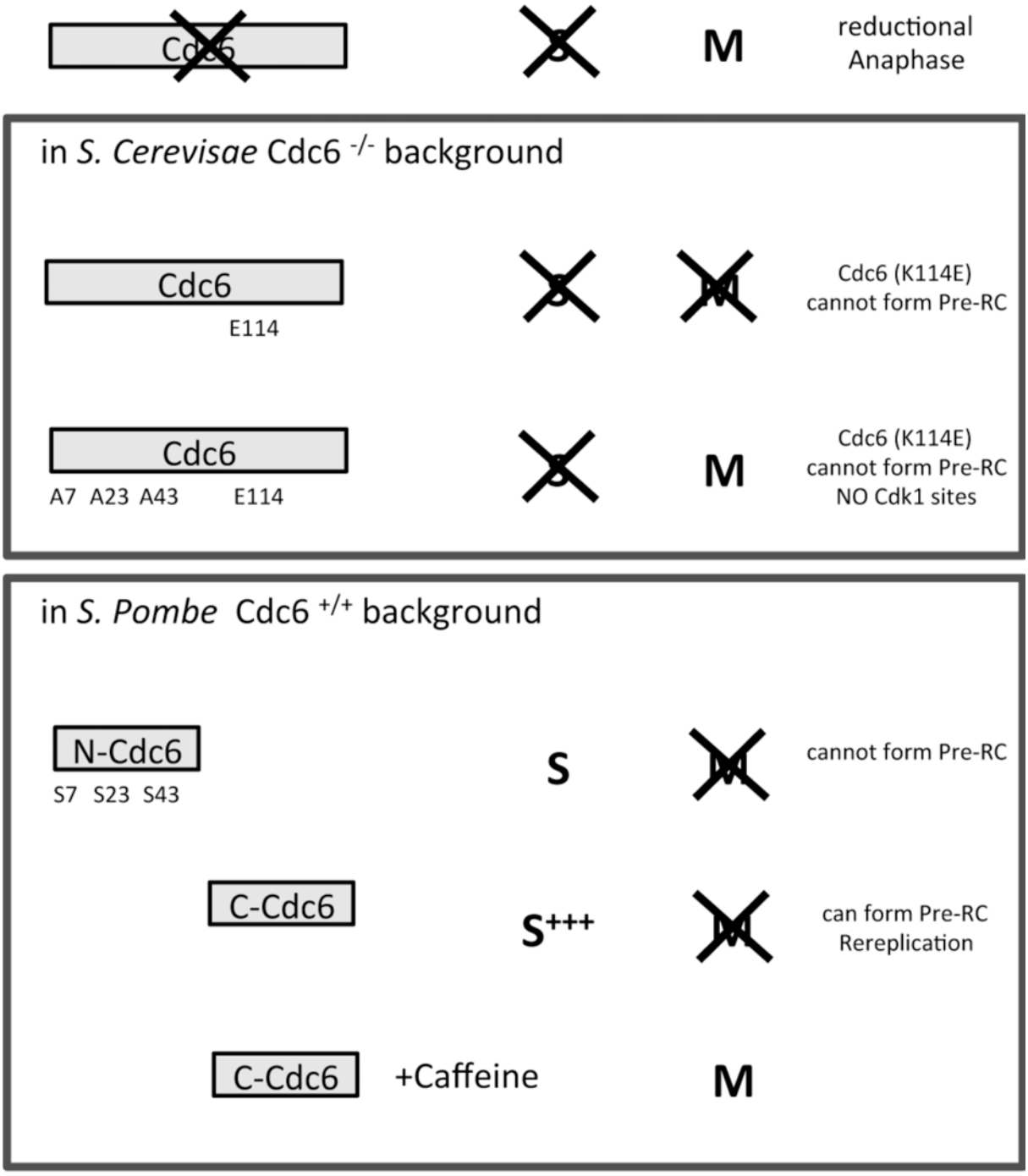

Fig. 36: The ability of Cdc6 to regulate M-phase entry

In yeast and mammals, Cdc6 depleted cells undergo mitosis without DNA replication. In yeast, Cdc6 modulates Cdk1 activity by directly interacting with Cdk1-Cyclin B through its N-terminus and/or by activating a checkpoint mechanism. Both mechanisms are sufficient to inhibit Cdk1-Cyclin B activation and M-phase entry.

walker A box and unable to support the loading of MCM despite its binding to DNA. In Cdc6-null cells, the Cdc6-K114E overexpression still inhibits the G_2/M transition, as expected. In this context, the further removal of Rad53 (Chk2 homologues) does not rescue the entry in M-phase (Weinreich *et al.* 2001). Importantly, the expression of Cdc6-K114E-ΔN-terminus, deleted for the N-terminus of Cdc6, is not longer able to block M-phase entry (Weinreich *et al.* 1999). This N-terminus fragment of Cdc6 contains three sites of phosphorylation for Cdk1 at T7, T23 and S43 and their mutation in Alanine (Cdc6-K114E-T7A/T23A/S43A) make Cdc6 unable to block cells in G_2 (Weinreich *et al.* 2001). Since Cdc6-K114E-T7A/T23A/S43A does not interact with Cdk1-Cyclin B, these results nicely demonstrate that Cdc6 blocks the G_2/M transition by interacting and directly inhibiting Cdk1 activation and that the mitotic Cyclins and the N-Terminus of Cdc6 are both fundamental for this process. This block of the G_2/M transition induced by the overexpression of Cdc6 mutants unable to support DNA replication is independent of the activation of checkpoint mechanisms (**Fig. 36**).

On the other hand, in *S. Pombe*, the overexpression of wild-type CDC18 (Cdc6 homolog) or mutants CDC18 that support DNA replication restricts the entry into M-phase but also induces multiple round of DNA replication, thus generating cells with DNA content higher than 2N (Nishitani *et al.* 1995). This phenotype can be explained if overexpressed Cdc6 allows multiple round of licensing of replicative origins and if simultaneously the M-phase entry is inhibited, escaping the reduction of the DNA content by half. Several Cdc6 mutants were then overexpressed in *S. Pombe* and their ability to induce re-replication and to block M-phase entry was followed by monitoring DNA content and analysing cellular morphology (Greenwood *et al.* 1998). The overexpression of the N-terminus of Cdc6 neither induce DNA re-replication, nor support pre-RC assembly, but blocks Cdk1 activity as seen by the elongation of the cell due to the absence of division (Greenwood *et al.* 1998). This effect is independent of checkpoint mechanisms as the presence of caffeine does not restore cell division (Greenwood *et al.* 1998). As mentioned before, the N-terminus of Cdc6 contains the three sites essential for the binding to Cdk1 (Weinreich *et al.* 2001) that certainly directly inhibit Cdk1. On the other hand, overexpressing the C-terminus of Cdc6 induces DNA re-replication and inhibits the M-phase occurrence (Greenwood *et al.* 1998). This mutant cannot directly bind and inhibit Cdk1 and blocks M-phase entry by inducing the activation of a caffeine-sensitive checkpoint (Greenwood *et al.* 1998) (**Fig. 36**).

In summary, the absence of Cdc6 in yeast blocks DNA replication and allows the cell to undergo a reductional anaphase. Cdc6 inhibits Cdk1 through two independent pathways. The first one involves the N-terminus of the protein that directly interacts with Cdk1 and inhibits its activity. The second one is promoted by the C-terminus of Cdc6 and activates of a checkpoint mechanism that indirectly restrains Cdk1 activation, inhibiting the G_2/M transition. However, the C-terminus of Cdc6 can

support DNA replication and induces re-replication when overexpressed, in *S. Pombe*. It will be interesting to mutagenize the ATP-binding site in this fragment (C-terminus-ΔATP) to determine whether this fragment of Cdc6 can still induce the activation of the checkpoint. If overexpressing C-terminus-ΔATP does not induce checkpoint activation, it would strongly suggest that the activation of the checkpoint induced by the C-terminus of Cdc6 relies on the process of re-replication in *S. Pombe*.

ii. Role of Cdc6 in higher eukaryotes

The role of Cdc6 in coordinating S-phase with M-phase is conserved in mammals. The depletion of Cdc6 from HeLa cells prevents DNA replication but these cells still proceed through M-phase, without activating a checkpoint mechanism (Lau *et al.* 2006). The overexpression of human Cdc6 also blocks the entry in M-phase in HeLa cells but, on the contrary to yeast, this inhibition cannot be rescued by overexpressioning Cdk1-Cyclin B (Clay-Farrace *et al.* 2003). This result suggests that the direct interaction between Cdc6 and Cdk1 is not responsible for the arrest at the G_2/M transition induced by overexpressed Cdc6. However, the entry into M-phase can be restored by overexpressing either a constitutive active form of Cdk1, Cdk1 (T14A/Y15A)-Cyclin B, Cdc25B or Cdc25C (Clay-Farrace *et al.* 2003). These experiments suggest that the cell cycle arrest is caused by a checkpoint cascade that leads to the inactivation of Cdc25. Surprisingly, this checkpoint induced by human Cdc6 is not inhibited by Caffeine and M-phase entry is restored using a selective inhibitor of Chk1, UCN-01 (Clay-Farrace *et al.* 2003). This suggests that Cdc6 would induce the activation of Chk1 independently of ATM and ATR activities.

In *Xenopus* interphasic extracts, Cdc6 is released from the replicative origins after their licensing and is again recruited onto the replication forks following their slicing a few hundred base pairs away from the replicative origin (Oehlmann *et al.* 2004). The binding of Cdc6 to the replicative fork depends on ORC proteins. This binding is required to induce the ATM/ATR-dependent phosphorylation of Chk1 in response to stalled replicative forks caused by the addition of Aphidicolin (Oehlmann *et al.* 2004). This function of Cdc6 in activating a checkpoint following replicative damages and leading to the arrest of the cell in S-phase is conserved in fission yeast. Upon Hydroxyurea (HU) exposure, *S. pombe* cells get arrested in S-phase. Under this condition, the expression level of Cdc6 as well as its binding to the chromatin are stabilized, allowing the recruitment of ATR (Hermand *et al.* 2007). The depletion of Cdc6 promotes the release of ATR from chromatin and the entry in aberrant mitosis (Hermand *et al.* 2007). The interaction between Cdc6 and ATR and their involvement in the replicative checkpoint was confirmed in human cells and in *Xenopus* using extracts (Yoshida *et al.* 2010).

In conclusion, the depletion of Cdc6 in mammalian cells blocks DNA replication and promotes M-phase as in yeast. However, on the contrary to yeast, the overexpression of Cdc6 does not directly inhibit Cdk1 activity but rather prevents Cdk1 activation through a checkpoint mechanism. This mechanism is certainly the physiologically one that surveys DNA replication in the presence of stalled forks. Cdc6 is unlikely to induce re-replication as its overexpression is not sufficient for launching the re-replication and additional components must be switched-off to promote the re-replication process (Siddiqui *et al.* 2013). Therefore, Cdc6 overexpression blocks entry in M-phase by activating the checkpoint pathway surveying the presence of stalled forks.

The direct effect of Cdc6 on Cdk1 seems to be conserved only in lower eukaryotes. However, an effect of Cdc6 on M-phase entry has been recently reported in *Xenopus* cycling extracts (El Dika *et al.* 2014). In extracts treated with Norcantharidin, which decreases the level of Cdc6 expression (Li *et al.* 2006), or supplemented with a blocking antibody against Cdc6, Cdk1 activation was advanced (El Dika *et al.* 2014). Reversely, the addition of recombinant Cdc6 delayed Cdk1 activation that is restored following the overexpression a non-degradable form of Cyclin B. This suggests that in *Xenopus*, Cdc6 has the potential to directly inhibit Cdk1-Cyclin B complexes during the first embryonic cell cycle, as it does in yeast (El Dika *et al.* 2014).

b. Cdc6 and the progression through M-phase

i. Cdc6 and the spindle formation

While in yeast Cdc6 is rapidly degraded after S-phase, Cdc6 is present in G_2- and S-phases during the mammalian cell cycle and its function is regulated by changes in its subcellular localization (Williams *et al.* 1997). Human Cdc6 is nuclear in G_1 and is translocated to the cytoplasm at the beginning of S-phase via a Crm1-dependent export mechanism (Petersen *et al.* 1999) (Jiang *et al.* 1999). In CHO cells, overexpressed Cdc6 localizes to the mitotic spindle (Illenye *et al.* 2004). Human cells depleted for Cdc6 in S-phase enter in mitosis, despite the lack of DNA synthesis, but the spindle is abnormally formed leading to aberrant chromosomal congression (Lau *et al.* 2006). Altogether, these results suggest a role for Cdc6 in the spindle formation.

This role is conserved in mouse and *Xenopus* oocytes (Anger *et al.* 2005, Narasimhachar *et al.* 2012). As in *Xenopus* oocytes, Cdc6 is not present in prophase-arrested mouse oocytes. The protein is barely detected at GVBD and accumulates starting MI to reach a significant level at MII (Lemaitre *et al.* 2002, Whitmire *et al.* 2002, Lemaitre *et al.* 2004, Anger *et al.* 2005). In mouse oocytes, the selective destruction of Cdc6 mRNA by injecting dsRNA does not block meiosis resumption as seen by Cdk1 activation, chromosomes condensation and nuclear envelope breakdown but the spindle does not form and the 1st polar body is not extruded (Anger *et al.* 2005). In *Xenopus* oocytes, a small amount

of Cdc6 starts to be detected shortly after GVBD by immunofluorescence. It localizes around the spindle precursor that forms at the basis of the germinal vesicle and then to the spindle poles of both MI and MII spindles (Narasimhachar *et al.* 2012). The inhibition of Cdc6 expression with antisense oligonucleotides or its inhibition by injecting blocking antibodies strongly perturbs the formation of the spindle as in mouse oocytes and promotes the appearance of microtubules monasters (Narasimhachar *et al.* 2012). Furthermore, the rotation of the spindle and its attachment to the animal cortex are abolished. The precise role of Cdc6 in the formation of the spindle remains elusive but Cdc6 could act as a structural protein independently of its role as a replicative factor.

ii. The control of the metaphase to anaphase transition

In budding yeast, the exit from M-phase is controlled by the activation of APC^{Cdc20} that specifically promotes the degradation of the securin, Pds1, and then by $APC^{Hct1/Cdh1}$ that controls the proteolysis of the mitotic Cyclins (Schwab *et al.* 1997, Visintin *et al.* 1997, Shirayama *et al.* 1998). Interestingly, cells missing Hct1/Cdh1 are unable to degrade mitotic Cyclins at the onset of anaphase but still, the activity of Cdk1 decreases and the cells exit from mitosis with a moderate delay (Schwab *et al.* 1997, Calzada *et al.* 2001). The mechanism controlling the exit from M-phase independently of Cyclin degradation relies on proteins that directly inhibit Cdk1 activity (Schwab *et al.* 1997). However, the substitution at the end of mitosis of the encoding Cyclin B gene with a non-degradable form of Cyclin B (Cyclin B-ΔDbox) block the cells in M-phase (Wasch *et al.* 2002). It was then proposed that impairing the Hct1/Cdh1-dependent degradation of Cyclin B is not sufficient to block the exit from M-phase because of the APC^{Cdc20} -dependent degradation of Cyclin B through the D-box. However, the expression level of Cyclin B-ΔDbox is much higher than the endogenous wild-type Cyclins (Wasch *et al.* 2002) and could result in a non-physiological hyperactivation of Cdk1 insensitive to the Cdk1 direct inhibitors, thus causing the M-phase arrest. These experiments shed new light on the M-phase control by direct Cdk1 inhibitors.

Several direct inhibitors of Cdk1 are known, as including Sic1 (the yeast homolog of $p27^{Cip1}$) and Cdc6. Cdc6 can inhibit Cdk1 activity independently of Sic1. The Cdc6 inhibitory effect toward Cdk1 depends on the first 47 residues of the N-terminus of Cdc6 since the expression of (Δ47)-Cdc6 (truncation of these 47 residues) still supports DNA replication without inhibiting Cdk1 activity (Calzada *et al.* 2001). Cell lines either expressing (Δ47)-Cdc6 instead of wild-type Cdc6 or depleted for Sic1 (Δsic1) are able to exit M-phase normally as seen by the degradation of mitotic Cyclins and Cdk1 inactivation (Calzada *et al.* 2001). These results prove that neither Cdc6 nor Sic1 are necessary for the metaphase to anaphase transition. However, cells combining both mutations, (Δ47)-Cdc6 and Δsic1, are blocked

with two nuclei containing extended mitotic spindles (Calzada *et al.* 2001), arguing for a defect in late anaphase. These results were challenged by another group that produced a viable Δ47-Cdc6 cell line performing full mitosis (Archambault *et al.* 2003). Therefore, whether the presence of CDK direct inhibitors is required for the metaphase to anaphase transition in yeast remains controversial. However, other evidence supports a role of Cdc6 in this process. Yeast cells depleted for Hct1/Cdh1 together with Cdc6 are neither able to degrade mitotic Cyclins nor to inhibit Cdk1 activity (Calzada *et al.* 2001). Moreover, a moderated overexpression of either Cdc6 or Sic1 rescue M-phase exit in Cyclin B-ΔDbox cell lines, which are blocked in M-phase with a high level of non-degradable mitotic Cyclins (Wasch *et al.* 2002)(Archambault *et al.* 2003). Therefore, the ability of Cdc6 in inhibiting Cdk1 is necessary for the exit from M-phase when the degradation of mitotic Cyclins is prevented, such as in ΔHct1/Cdh1 cell lines or in Cyclin B-ΔDbox cell lines. Under these conditions, Cdk1 inhibitors, as Sic1 or Cdc6, can inhibit the large number of active and stable CDK-Cyclin complexes and allow the exit from M-phase.

C. Cdc6 in apoptosis

Apoptosis is an organized program leading to cell death in response of internal stress stimuli (intrinsic pathway) or external receptors (extrinsic pathway). The intrinsic pathway involves the formation of mitochondrial pores, which derive from the polymerization of Bax and Bak proteins and permeabilize the outer membrane of the mitochondria. This promotes the release in the cytoplasm of the Cytochrome C (CytC) that binds the protein Apaf-1 to form the heptameric complex called apoptosome. The apoptosome recruits and activates Caspase-9 that in turn activates Caspase-3, responsible for the execution of the apoptotic program. Interestingly, the expression of Apaf-1 is under the control of E2F transcription factor family that also regulates the expression of genes necessary for the S-phase execution. Among them is Cdc6.

When Cdc6 is overexpressed in fibroblasts in which apoptosis is induced, Cdc6 associates with monomeric Cyt C-Apaf-1 complexes, blocks the apoptosome and impairs the activation of Caspase-9 (Niimi *et al.* 2012). This function depends on the ATPase activity of Cdc6 since a mutant unable to hydrolyse ATP cannot bind CytC-Apaf-1 (Niimi *et al.* 2012). Therefore, endogenous Cdc6 could attenuate the apoptosis pathway in unstressed conditions (**Fig. 37**).

However, inhibiting the topoisomerase with etoposide, a treatment activating the intrinsic apoptotic pathway, induces the cleavage of Cdc6 by Caspase-3 leading to the production of a fragment of 49 kDa (p49-tCdc6) (Yim *et al.* 2003). This fragment localizes mainly in the nucleus and its expression not only enhances apoptosis, when activated by etoposide, but also induces apoptosis in unstressed HeLa cells (Yim *et al.* 2003). On the contrary, the expression of a Cdc6 mutant that cannot be cleaved

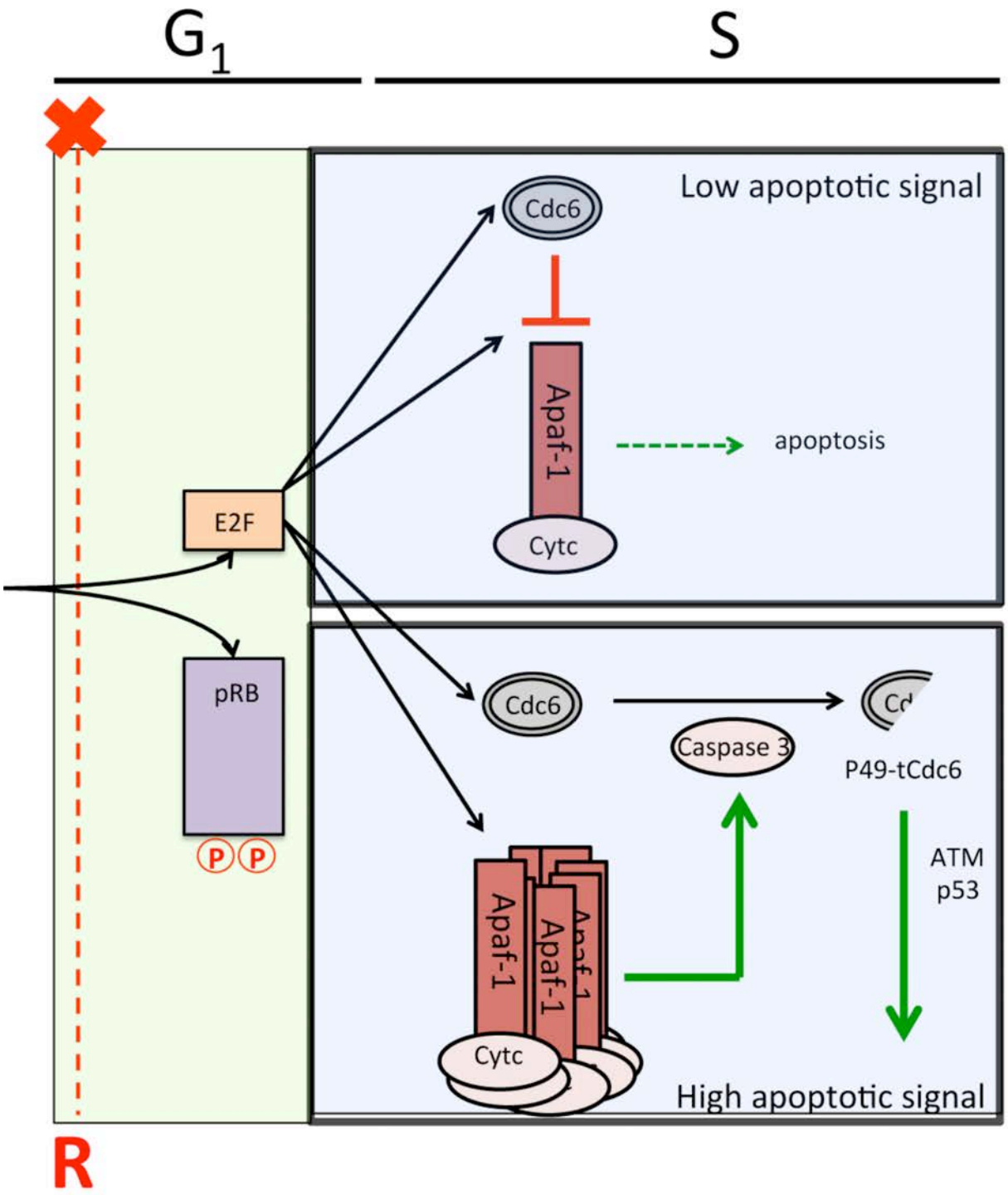

Fig. 37: Cdc6 modulates Apoptosis

During S-phase, both Cdc6 and Apaf-1, a pro-apoptotic protein, are expressed. In the presence of low apoptotic signal, Cdc6 inhibits the formation of the apoptosome by interacting with Apaf1-Cytochrome C complex. However, when the apoptotic signals is turned on, the apoptosome is formed and promotes the cleavage of Cdc6 cleavage, generating a truncated fragment fof Cdc6 (p49-tCdc6). P49-tCdc6 is translocated to the nucleus and acts as a dominant negative to block Pre-RC assembly. This activates a ATM/p53-dependent pathway that reinforces the apoptotic signal.

reduces the efficiency of Cdc6 in inducing apoptosis (Yim *et al.* 2006). The p49-tCdc6 fragment acts as a dominant negative mutant for the assembly of the pre-RCs by interfering with the loading of MCM_{2-7} on the chromatin (Yim *et al.* 2006). As MCM_{2-7} cannot be recruited on chromatin, ATM is activated and phosphorylates p53 at S15 causing the activation of a checkpoint that ends with the induction of apoptosis (Yim *et al.* 2006) (**Fig. 37**).

Altogether, Cdc6 appears as a modulator of apoptosis that represses this pathway under unstressed conditions but acts as an enhancer of the ATM- and p53-dependent pathway when an apoptotic signal is launched through its cleaved fragment.

D. Roles of Cdc6 during the meiotic division

a. The binding of oocyte to sperm

At the time of ovulation, the oocyte is released from the ovary and simultaneously resumes meiosis. In amphibian, the passage through the oviduct is necessary for the MII-arrested oocyte to acquire the ability to bind sperm. This process is mediated by a protease that is secreted by the oviduct and modifies the glycoprotein gp43 present in the coelomic egg envelop (CE). This protease converts the CE into the sperm-receptive vitelline envelope (VE) (Tian *et al.* 1997a). Prophase-arrested oocytes are unable to bind sperm despite their passage through the oviduct (Tian *et al.* 1997b). The injection in prophase oocytes of the total fraction of polyadenylated mRNAs extracted from MII-arrested oocyte does not induce meiotic maturation but confers to the oocyte the ability to bind sperm after the passage through the oviduct (Tian *et al.* 1997b). This suggests that a protein synthesized during meiotic maturation in response to the polyadenylation of its mRNA is required for the interaction between sperm and oocytes. After screening hundreds of mRNAs, it was found that Cdc6 mRNA is able to confer sperm binding ability (Tian *et al.* 1997b). This unexpected and surprising new role of Cdc6 has never been further explored since that time.

b. The competence to replicate

As described in the introduction, oocytes arrested in prophase I and quiescent cells arrested in G0 have developed a common strategy to avoid unscheduled DNA replication during their long lasting arrest. In both systems, the inability to replicate is due to the absence of the Cdc6 protein (Stoeber *et al.* 1998, Cook *et al.* 2002, Lemaitre *et al.* 2002, Whitmire *et al.* 2002, Lemaitre *et al.* 2004, Duursma *et al.* 2005, Mailand *et al.* 2005). When the cell re-enters the cell cycle, either in G_1 for somatic cells or in the 1st meiotic division for female germ cells, the ability to replicate is restored by the

accumulation of Cdc6 (Stoeber *et al.* 1998, Cook *et al.* 2002, Lemaitre *et al.* 2002, Whitmire *et al.* 2002, Lemaitre *et al.* 2004, Duursma *et al.* 2005, Mailand *et al.* 2005). In *Xenopus* oocyte, the Cdc6 protein starts to accumulate 45 to 60 minutes after GVBD and renders the replicative machinery functional. However, the oocyte progresses directly from the 1st to the 2nd meiotic division without using this functional replicative machinery (See chapter: I-B.e.iii. "The molecular control of DNA replication")

c. Working hypothesis: how to suppress the Cdc6- dependent synchronisation of S-phase with mitosis during meiotic maturation

During the mitotic cell cycle, an important role of Cdc6 is to synchronize S-phase with the entry into mitosis, a process fundamental to maintain genome stability and the ploidy of somatic cells. During meiosis, the ploidy must be reduced by half, what is achieved by the two successive divisions without intervening S-phase, each division being triggered by a wave of Cdk1 activation. During this meiosis-specific critical period, Cdc6 starts to be expressed and the replicative machinery becomes functional. Still, replication has to be repressed and the negative regulation exerted by Cdc6 on Cdk1 activation, if any, has to be suppressed to ensure the proper progression through the two meiotic divisions. One way to avoid this inhibitory effect could be by controlling precisely the timing of Cdc6 accumulation during meiotic maturation.

Little is known regarding the regulation of Cdc6 during meiotic maturation. Cdc6 is not expressed in prophase and the timing of its translation is unknown. Based on the polyadenylation of its mRNA, it has been proposed that Cdc6 could be synthesized around the time of GVBD. However, the protein starts to accumulate 45 minutes after GVBD. The delay between the initiation of its supposed translation and its accumulation, strongly suggests that Cdc6 accumulation and turnover are controlled by post-translational mechanisms in the oocyte.

E. Results

a. Generation of molecular tools

We started this project by cloning *Xenopus* wild-type Cdc6 (WT-Cdc6) to generate mRNA and recombinant GST-tagged proteins. We further generated by mutagenesis two mutants of Cdc6: a non-recognizable form of Cdc6 by SCF^{Cdc4} by mutating the F box TPxxS into APxxS (APxxS-Cdc6), and a non-phosphorylatable form of Cdc6 by mutating the 7 putative SP sites into alanine (7A-Cdc6). To further analyse Cdc6 in oocytes, we produced Cdc6 antibodies using *Xenopus* WT-Cdc6 as antigen (**Fig. 39**).

b. Cdc6 is stable in prophase and is degraded in a SCF^{Cdc4}-dependent-manner in response to progesterone

By injecting prophase oocytes with mRNA encoding WT- or APxxS-Cdc6, I discovered that Cdc6 is expressed in prophase. However, progesterone leads to the disappearance of the WT protein whereas the APxxS mutant is still expressed until the time of GVBD. Both proteins are then re-accumulated at GVBD until MII. These results suggest that the protein is degraded in response to progesterone in a SCF^{Cdc4}-dependent manner. The injection of recombinant WT- and APxxS-Cdc6 proteins confirmed this conclusion. To further characterize the degradation mechanism targeting Cdc6, the activity of the proteasome as well as Cdk1 activation were respectively inhibited using MG132 or $p21^{Cip1}$. Under both conditions, the degradation of WT-Cdc6 normally triggered by progesterone was abolished, arguing that progesterone activates a SCF^{Cdc4}-dependent mechanism that targets Cdc6 to the proteasome and induces its degradation. This process depends on Cdk1 activation, explaining why it happens just before GVBD, precisely at the time when Cdk1 activation is initiated.

c. Ectopic Cdc6 is turning-over during the MI-MII transition

Next, WT-Cdc6 and APxxS-Cdc6 were injected in oocytes at the time of GVBD, when the activation of Cdk1 is established. Both recombinant proteins remained detectable at a constant level during the entire process of meiotic maturation: during the MI-MII transition until the MII arrest. However, the inhibition of protein synthesis with CHX at GVBD led to the disappearance of both proteins within one hour. These results show that Cdc6 is subjected to turn-over during the MI-MII transition, being regulated by two opposite mechanisms: one promoting its stabilisation, which depends on protein synthesis, and a second one leading to its degradation, which is independent of SCF^{Cdc4}.

d. The accumulation of endogenous Cdc6 is under the dual control of Cdk1-Cyclin B and the Mos/MAPK pathway during the MI-MII transition

Since the addition of CHX at GVBD leads to the inactivation of the Mos/MAPK pathway and prevents the reactivation of Cdk1, we wondered whether these two kinase activities could play a role in regulating Cdc6 turnover. Both activities phosphorylate *in vitro* Cdc6 at SP sites and the mutation of these sites in alanine abolishes the phosphorylation of Cdc6 induced by either active Cdk1 or MAPK. Furthermore, the 7A mutant of Cdc6 is still degraded when injected at GVBD in the presence of CHX indicating that the phosphorylation of Cdc6 might not be involved in the stabilization and in the degradation of the protein post-GVBD.

To decipher the effects of Cdk1 and of MAPK on Cdc6 turnover, the activity of either Cdk1 or the Mos/MAPK pathway were inhibited after GVBD using either antisense oligonucleotides specifically directed against either Cyclin B mRNA or Mos-morpholinos oligonucleotides. These treatments do not block the 1st wave of Cdk1 activation and GVBD. However, oocytes are unable to reactivate Cdk1 in the presence of Cyclin B antisense whereas in the presence of Mos-morpholinos, the activity of Cdk1 reincreases after meiosis I. The accumulation of endogenous Cdc6 was monitored under each condition. In control oocytes, the accumulation of Cdc6 starts 45 minutes after GVBD. In Cyclin B antisense-injected oocytes, Cdk1 is not reactivated and the accumulation of Cdc6 is strongly delayed, occurring only 180 minutes after GVBD, at the time of MAPK inactivation. In contrast, in Mos-morpholinos oocytes, Cdc6 accumulation is advanced, being detected right after GVBD. Therefore, during the MI-MII transition, the accumulation of endogenous Cdc6 is positively regulated by Cyclin B accumulation or/and Cdk1 re-activation, whereas the Mos/MAPK pathway postpones the appearance of Cdc6. To decipher whether the Cyclin B-Cdk1 dependent stabilization of Cdc6 after GVBD is caused by Cyclin B accumulation or by the catalytic activity of Cdk1, recombinant WT-Cdc6 was injected at GVBD in oocytes treated with CHX together with trace-amounts of a non-degradable form of Cyclin B. This low amount of Cyclin B is not high enough to reactivate Cdk1 in CHX-treated oocytes. However, it abolishes the degradation of WT-Cdc6 in CHX-treated oocytes. Therefore, Cdc6 is not stabilized by the catalytic activity of Cdk1 after GVBD, but by the accumulation of Cyclin B. Since both endogenous and ectopic Cdc6 are able to interact with Cdk1-Cyclin B complexes, this further suggests that the direct interaction between Cdc6 and Cyclin B is involved in this stabilization mechanism, a process reminiscent of what happens the yeast system.

e. Overexpressing Cdc6 blocks Cdk1 reactivation and drives the oocyte into a replicative interphasic state after meiosis I

We then addressed the question of the consequences of the tight regulation of Cdc6 accumulation during meiotic maturation. Since Cdc6 is a potent inhibitor of Cdk1 in somatic cells as well as in *Xenopus* extracts, this led us to hypothesize that deregulating the expression level of Cdc6 could affect the activity of Cdk1 in our model system. In the experiments described previously, ectopic Cdc6 was expressed at a low concentration, 0.05 µM. When injected at this concentration either in prophase or at GVBD, Cdc6 affects neither entry into MI nor the reactivation of Cdk1 at MII entry. We then raised the amount of injected Cdc6 to 0.5 µM, corresponding to the physiological concentration of endogenous Cdc6 at MII. Surprisingly, the injection of this higher amount of WT-Cdc6 delays GVBD induction and decreases the level of Cdk1 activity in response to progesterone. This inhibitory effect over Cdk1 activation is enhanced by the APxxS mutation that stabilizes Cdc6: almost all the oocytes injected by this stable mutant are unable to activate Cdk1 and to resume meiosis. We assume that the APxxS-Cdc6 is more efficient because it is not degraded upon progesterone addition. Therefore, Cdc6 can inhibit Cdk1 activation and entry into the 1st meiotic division. Next, 0.5 µM of WT-Cdc6 was injected at GVBD and the reactivation of Cdk1 was followed up to 4 hours after GVBD. Injected WT-Cdc6 remains stable after GVBD and abolishes the reactivation of Cdk1 as well as the entry into meiosis II. Under this condition, the oocytes replicate their DNA. Importantly, the inhibition of Cdk1 induced by Cdc6 is still observed when DNA replication is blocked by aphidicolin. This indicates that Cdc6 directly inhibits Cdk1 activation and that this process is not an indirect effect caused by DNA replication. Conversely, the setting-up of DNA replication directly depends on the inhibition of Cdk1 induced by Cdc6.

f. A new model for the regulation of Cdc6 during the meiotic divisions

Based on these results, we propose a model wherein the tight control of Cdc6 accumulation is critical to ensure the proper activation of Cdk1 and thus, the succession of the two meiotic divisions with no intervening S-phase.

In this model, the translation of Cdc6 is initiated at the time of the 1st Cdk1 activation. However, the protein cannot accumulate because Cdk1 activates a SCF^{Cdc4}-dependent mechanism that targets Cdc6 for degradation to the proteasome. While this mechanism may not be physiologically involved during meiosis resumption, it could be seen as a safeguard mechanism to ensure the absence of Cdc6 at the time of the 1st activation of Cdk1 that could otherwise perturb meiosis resumption. Starting from

GVBD, Cdc6 cannot accumulate at high levels because the Mos/MAPK pathway is activated, promoting the degradation of the protein, and because Cyclin B, that stabilizes Cdc6, is rapidly degraded once MI is completed. One hour after GVBD, Cyclin B starts to reaccumulate and stabilizes Cdc6 through a direct interaction. This stabilization mechanism overcomes the negative effect exerted by the Mos/MAPK pathway on Cdc6 turnover and allows the progressive accumulation of Cdc6 during the entry into MII and during the CSF arrest. This tight regulation controls the expression level of Cdc6 during the MI-MII transition and prevents its ability to inhibit Cdk1 activity, which would lead to DNA replication. This process then indirectly inhibits the occurrence of DNA replication between the two meiotic divisions but allows at the same time the accumulation of Cdc6 that is necessary after fertilization to support the embryonic cell cycles.

F. Discussion

During the mitotic cell cycle, S- and M-phases are synchronized to ensure that the cell division does not start before the completion of DNA replication. In proliferative cells, one way to arrest the cell cycle before M-phase is to inactivate or to remove pre-RCs. When unsynchronized cells are treated with aphidicolin, replication does not start and cells accumulate at the G_1/S (Pedrali-Noy *et al.* 1980). In yeast, the expression of a temperature-sensitive mutant of ORC5 inhibits DNA-replication as expected, but also blocks the cell cycle either at the G_1/S transition or in early M-phase (Dillin *et al.* 1998), whereas the expression of temperature-sensitive mutants of Mcm2 and Mcm3 arrests the cells before M-phase (Yan *et al.* 1991). These phenotypes imply that these proteins have additional functions during the cell cycle than regulating DNA replication in S-phase. Surprisingly, the depletion of either Cdc6 or Cdt1 abolishes DNA replication in yeast, but the cells are still entering and completing mitosis and, consequently, undergo a reductional anaphase (Hofmann *et al.* 1994, Piatti *et al.* 1995). By directly or indirectly inhibiting Cdk1 activation in this model system, Cdc6 is therefore able to synchronize S-phase with M-phase, avoiding the reduction of the ploidy and contributing to maintain genome stability.

During meiosis, this synchronization must be lost to ensure the succession of two meiotic divisions without an intervening S-phase. This process is necessary to reduce by half the ploidy of the cell and to generate fertilizable haploid gametes. In *Xenopus* oocytes, we show that Cdc6 has the potential to inhibit Cdk1 activity by directly interacting with Cdk1-Cyclin B complexes, independently of the activation of ATM and ATR-dependent checkpoints. This result strongly suggests that the function of Cdc6 as a synchronizer of S-phase with cell division is conserved during meiotic maturation of *Xenopus* oocytes, although this function is repressed. The tight control of the timing of Cdc6 accumulation in oocytes is used as a strategy to ensure the inhibition of DNA replication without arresting the cell between the two meiotic divisions.

a. A tiny window between the initial activation of Cdk1 and GVBD to get rid out Cdc6

During the prophase arrest, the absence of Cdc6 is sufficient to prevent the assembly of the pre-RCs (Lemaitre *et al.* 2002, Whitmire *et al.* 2002) (**Fig. 38**). As the oocyte resumes meiosis, Cdc6 mRNA are polyadenylated downstream the 1st activation of Cdk1, suggesting that Cdc6 translation is initiated around the time of GVBD (Lemaitre *et al.* 2002). We confirmed this Cdk1-dependent regulation by

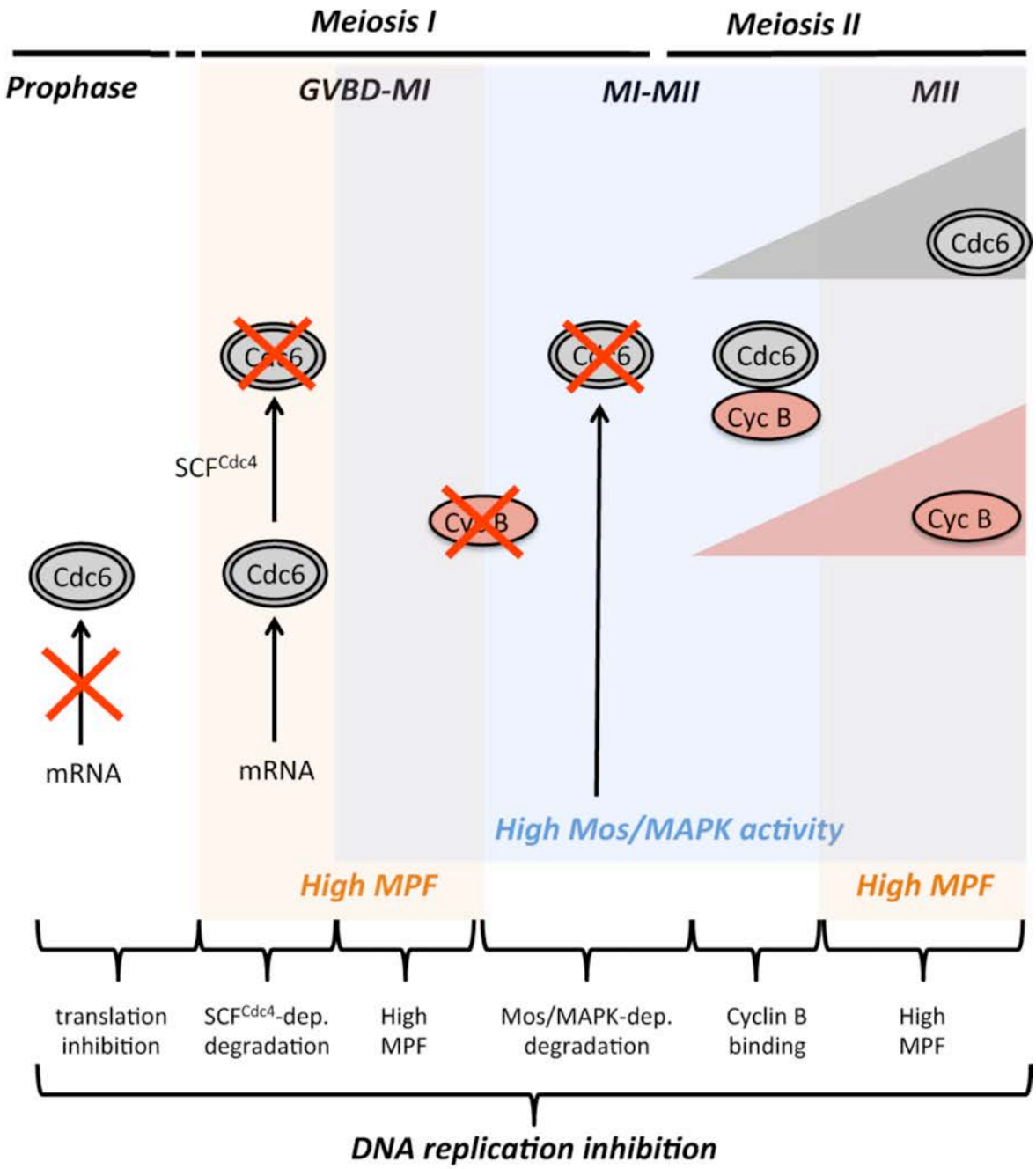

Fig. 38: Cdc6 is tightly regulated to guarantee the inhibition of DNA replication during meiotic maturation of *Xenopus* oocytes

During the long lasting prophase arrest, DNA replication is inhibited due to the absence of Cdc6. During meiosis resumption and downstream Cdk1 activation, the translation of Cdc6 is initiated but the protein cannot accumulate because of the activation of a SCF^{Cdc4}-dependent degradation. In Metphase I, the high level of Cdk1 activity is sufficient to prevent DNA replication. In Anaphase I, the activity of Cdk1 decreases but the Mos/MAPK pathway still prevents Cdc6 accumulation. Until this moment, the absence of Cdc6 is sufficient to explain the inhibition of DNA replication. Once Cyclin B starts to reaccumulate, Cdc6 interacts with Cdk1-Cyclin B and is stabilized. This interaction could prevent Cdc6 ability from assembling Pre-RC at that time when Cdk1 activity is low. Finally in MII, DNA replication is inhibited by the high Cdk1 activity.

showing that the inhibition of the 1st activation of Cdk1 by $p21^{Cip1}$ injection prevents the accumulation of endogenous Cdc6 in response to progesterone. However, the protein cannot accumulate because a degradation mechanism is simultaneously activated and targets Cdc6 to the proteasome for proteolysis. This degradation mechanism relies on the F-box binding motif of Cdc6, and thus certainly on the SCF^{Cdc4} system, and is activated in a Cdk1-dependent manner. Whether this SCF^{Cdc4} degradation mechanism has a physiological role and/or is necessary during meiosis resumption is unknown. However, it is interesting to note that Cdc6 is not the only regulator of Cdk1 that is degraded by this system. However SCF ubiquitination system was already reported to be active during meiosis resumption since Erp1 is degraded by SFC^{Btrcp} when overexpressed in prophase oocytes (Margottin-Goguet *et al.* 2003). When prophase oocytes are injected with high amounts of WT-Cdc6, they resume meiosis and activate Cdk1, but after a significant delay. Moreover, the same amount of a stable form of Cdc6, which cannot be recognized by SCF^{Cdc4}, abolishes meiosis resumption and Cdk1 activation. This suggests that the SCF^{Cdc4}-dependent degradation system could act as a safeguard mechanism to eliminate right before GVBD any traces of Cdc6 that could disturb the full activation of Cdk1 (**Fig. 38**). One important issue regards the mechanism induced by Cdk1 and leading to Cdc6 degradation. Is the Cdk1-dependent phosphorylation of Cdc6 required for its recognition by SCF^{Cdc4} and its consecutive degradation, as in yeast (Drury *et al.* 1997, Perkins *et al.* 2001)? Is Cdk1 regulating directly the activity of SCF^{Cdc4}? The injection of the 7A-Cdc6 mutant in prophase will tell us whether the phosphorylation of Cdc6 at SP sites is necessary for its degradation in response to progesterone.

b. The Mos/MAPK pathway: a Godfather for Cdk1 between the two meiotic divisions

Once Cdk1 is fully activated, it induces the breakdown of the nuclear envelope and DNA condensation, two conditions incompatible with DNA replication in somatic cells. At GVBD, the Mos/MAPK pathway is also activated. When the activation of this pathway is prevented with Mos antisense oligonucleotides or U0126, the oocytes enter an interphasic state and replicate DNA (Furuno *et al.* 1994, Gross *et al.* 2000, Dupre *et al.* 2002). Therefore, the Mos/MAPK pathway plays a pivotal role in inhibiting DNA replication after the 1st meiotic division.

This kinase cascade (from Mos to $p90^{Rsk}$) positively regulates the level of Cyclin B expression during the MI-MII transition in two manners: by attenuating its degradation and by promoting its synthesis (Gross *et al.* 2000, Dupre *et al.* 2002). The Mos/MAPK pathway then contributes to Cdk1 reactivation by ensuring the fast reaccumulation of Cyclins B after the meiosis I, thus leading to entry into meiosis II. The fact that recombinant Mos injected at GVBD is unable to prevent the occurrence of DNA

replication in the presence of CHX strongly suggests that the ability of the Mos/MAPK pathway in inhibiting DNA replication acts through the activity of Cdk1 (Furuno *et al.* 1994).

However, our results enlighten a new function of the Mos/MAPK pathway that postpones the accumulation of Cdc6 after GVBD. The inhibition of the Mos/MAPK pathway with either Mos morpholino or U0126 strongly advances Cdc6 accumulation. Importantly, this new function of the Mos/MAPK pathway is independent of the level of Cyclin B expression, as oocytes injected with Cyclin B oligonucleotides cannot accumulate Cdc6 unless the Mos/MAPK pathway gets inactivated. Since the absence of Cdc6 is sufficient to avoid DNA replication between the two meiotic divisions, the Mos/MAPK pathway then can inhibit DNA replication on its own by delaying the accumulation of endogenous Cdc6, independently of its effect over Cyclin B turnover (**Fig. 38**). How the Mos/MAPK controls Cdc6 turnover and what are the mechanisms leading to the degradation of Cdc6 during the MI-MII transition deserve further investigation.

In mitosis, APC^{Cdh1} is active at late anaphase and promotes the degradation of Cdc6. Cdh1 is expressed in both prophase- and MII-arrested oocytes (Zhou *et al.* 2002, Papin *et al.* 2004). In *Xenopus*, since the inhibition of Cdh1 expression with antisense oligonucleotides prevents meiosis resumption induced by progesterone (Papin *et al.* 2004), its involvement during the MI-MII transition is difficult to address. In yeast, a specific inhibitor of APC^{Cdh1}, called Acm1, has been identified. This inhibitor acts as a pseudo-substrate by preventing the interaction of Cdh1 with its physiological substrates (Martinez *et al.* 2006, Martinez *et al.* 2012). Acm1 could be used in *Xenopus* oocytes to inhibit the function of Cdh1 after GVBD in order to determine whether this protein is involved or not in Cdc6 degradation during the MI-MII transition.

The other form of APC/C, APC^{Cdc20}, is activated at the time of GVBD and its inhibition with antisense oligonucleotides does not prevent the MI-MII transition (Taieb *et al.* 2001). Our preliminary data indicates that the inhibition of Cdc20 synthesis with antisense oligonucleotides advances the accumulation of Cdc6 compared to control oocytes. This suggests that the activity of APC^{Cdc20} could regulate the level of Cdc6 accumulation. Whether this mechanism is direct or is mediated through Cyclin B accumulation remains an important question to elucidate. Interestingly, in yeast, the cells can exit from M-phase independently of the degradation of mitotic Cyclins due to the presence of direct inhibitors of Cdk1 such as Cdc6 (See chapter: III-,B.b.ii. “the control of metaphase to anaphase transition”). Since oocytes accumulate Cdc6 in advance when the synthesis of Cdc20 is prevented, Cdc6 could counteract the absence of Cyclin B degradation during the exit from MI by inducing a local inhibition of Cdk1.

c. What roles for the interaction between Cdc6 and the Cyclins B ?

During the MI-MII transition, the reaccumulation of Cyclin B promotes the reactivation of Cdk1, the stabilization of Cdc6 and the oocytes enter meiosis II. Furthermore, both endogenous and recombinant Cdc6 directly interact with Cdk1-Cyclin B complexes. The activity of Cdk1, a potent inhibitor of DNA replication in somatic cells, increases concomitantly with Cdc6 because both events rely on Cyclin B accumulation. Therefore, Cdc6 starts to accumulate when Cdk1 is not yet fully reactivated. At that moment, the interaction between Cdc6, Cdk1 and Cyclin B would therefore support a double function: stabilizing Cdc6 (that needs to be accumulated to support the future embryonic cell cycles) and sequestering Cdc6 to block its ability to form pre-RC. This mechanism has already been proposed in yeast, in which the binding of Cdc6 to the mitotic Cyclins prevents Cdc6 from interacting with ORC proteins (Mimura *et al.* 2004).

This mechanism of "Cdc6 trapping" that both stabilizes Cdc6 and blocks pre-RCs formation is reminiscent of Cdt1 inhibition by Geminin. Geminin was shown to stabilize Cdt1 by preventing its ubiquitin-dependent degradation in G_2 and during mitosis (Ballabeni *et al.* 2004) and by further inhibiting its ability to form pre-RC (Ballabeni *et al.* 2013). At the end of anaphase, APC^{Cdc20} is activated, promotes the ubiquitination of Geminin that releases Cdt1. Cdt1 then participates in the licensing of replicative origins. This simplified model for Cdt1 regulation by Geminin has been challenged showing that Geminin binds Cdt1, but the effect of this interaction, in terms of promoting or inhibiting pre-RC formation, depends on the stoichiometric of Cdt1/Geminin complexes (Lutzmann *et al.* 2006).

In *Xenopus* oocytes, Cdt1 and Geminin are already present in prophase and their levels of expression increase significantly during meiotic maturation (Lemaitre *et al.* 2002, Whitmire *et al.* 2002, Narasimhachar *et al.* 2009). In this model system, Geminin stabilizes Cdt1 as seen by the decrease in the expression level of Cdt1 following the inhibition of Geminin synthesis. However, Geminin is not necessary to inhibit DNA replication in Xenopus oocytes, since its downregulation does not induce unscheduled DNA replication (Narasimhachar *et al.* 2009). Whether Geminin overexpression is sufficient to inhibit DNA replication induced by CHX treatment after GVBD is not known. These experiments suggest that Geminin, at its physiological concentration, may not be able to modulate Cdt1 ability to form pre-RC during *Xenopus* meiotic maturation. The situation of the oocytes after GVBD is therefore critical because there is no mechanism to restrict the pre-RC assembly beside the lack of Cdc6. Therefore, once Cdc6 start to accumulate, the interaction between Cdc6, Cdk1 and Cyclin B could become an essential mechanism preventing DNA replication initiation (**Fig. 38**). Upon fertilization, the degradation of Cyclin B releases Cdc6 that can in turn contribute to the pre-RC

formation. Cyclin B would therefore play two opposite roles on DNA replication during meiotic maturation: preventing the origin licensing during the MI-MII transition by sequestering Cdc6, but allowing its accumulation that is necessary to support DNA replication after fertilization, during the embryonic cell cycles.

To better understand Cdc6 stabilization by Cyclin B, it will be first important to determine whether Cdc6 interacts with Cdk1, with Cyclin B or both proteins by performing GST pull down experiments in oocytes depleted from Cyclin B by antisense oligonucleotides. In yeast, Cdc6 directly interacts with the mitotic Cyclins (Mimura *et al.* 2004). Recombinant Cdc6 is degraded when injected at GVBD in CHX-treated oocytes but remains stable when oocytes are further injected with recombinant non-degradable Cyclin B. This strongly suggests that the interaction between Cdc6 and Cyclin B is required for Cdc6 stabilization during the MI-MII transition. However, when Cdc6 is injected in prophase, progesterone provokes the degradation of Cdc6 despite the presence of pre-MPF and the synthesis of new Cyclin B molecules. This result suggests that the interaction between Cdc6 and Cyclin B either does not occur *in ovo* (GST-Cdc6 interacts with Cdk1-Cyclin B in prophase extracts) or is unable to protect Cdc6 from degradation before GVBD. Therefore, the regulation of Cdc6 stability may be differentially regulated during the lag period separating hormonal stimulation and GVBD on one hand, and the MI-MII transition on the other hand. Several mechanisms can account for these different regulations.

i. The phosphorylation status of Cdc6

At GVBD, both Cdk1-Cyclin B and the Mos/MAPK pathway are activated. Cdk1 and MAPK are able to *in vitro* phosphorylate Cdc6 and control the accumulation of the endogenous Cdc6 in an opposite manner. Whether the phosphorylation statute of Cdc6 could control directly its turnover or its interaction with Cyclin remains to be determined. The analysis of the *Xenopus* Cdc6 sequence indicates that Cdc6 contains 3 consensus sites (S/T)Px(K/R) for Cdk1, 2 Px(S/T)P motifs for MAPK and 2 others that can be potentially phosphorylated by both Cdk1 and MAPK (**Fig. 39**).

The mutation of all S within these SP sites into non-phosphorylatable A residues (7A-Cdc6) abolished the phosphorylation of Cdc6 by either Cdk1 or MAPK. When injected at GVBD, the 7A-Cdc6 mutant behaves exactly the same than WT-Cdc6, being stable in the absence of CHX and degraded in the presence of CHX. Therefore, the direct phosphorylation of Cdc6 at these 7 sites is unlikely involved in the stabilization/destabilization of the protein. However, since the Cdk1- and MAPK-dependent phosphorylations of Cdc6 are expected to have opposite effects on Cdc6 stability, the mutation into alanine of serines within these SP sites is probably not the best approach to investigate their

functions. Good tools to tackle this question would be to get Cdc6 mutants, where phosphorylation by one pathway is abolished while phosphorylation by the other one is preserved. I designed a mutant in which all 5 predicted SP sites for Cdk1 were suppressed without affecting the MAPK ones. Unfortunately, this mutant was still weakly phosphorylated by Cdk1 in our *in vitro* kinase assay. This results suggests that the putative sites phosphorylated by MAPK can be also targeted by Cdk1. Furthermore, since it is difficult to demonstrate whether these phosphorylations also take place *in vivo* without specific anti-phospho antibodies, we did not investigate further the regulation of this particular mutant of Cdc6 in the oocyte.

An interesting mechanism to control the stability and the ability of Cdc6 to bind Cdk1-Cyclin B has been described in human cells. During mitosis, the phosphorylation of Cdc6 at T37 by Plk1 was shown to regulate the interaction between Cdc6 and Cdk1-Cyclin B, contributing to the Cdc6-mediated inhibition of Cdk1 during the metaphase to anaphase transition (Yim *et al.* 2010). In *Xenopus*, Plx1, the homolog of Plk1, is activated at GVBD time and participates in the MPF automplification loop. The timing of Plx1 activation is compatible with the period when Cdc6 interacts with Cyclin B. However, human T37 is not conserved in *Xenopus* Cdc6. Another minimal consensus phosphorylation site for Plk1 ((E/D)x(S/T)(L/M/F/W/V/A/C/I) (Nakajima *et al.* 2003)) is located around S149 in *Xenopus* Cdc6 (**Fig. 39**). Whether Plx1 phosphorylates S149 during meiotic maturation is unknown but it would be interesting to test this hypothesis using purified Plx1 in our *in vitro* kinase assay. Moreover, it will be interesting to test the stability as well as the ability to block Cdk1 activation using a non phosphorylatable form of Cdc6 (S149A-Cdc6).

ii. The specific binding of Cdc6 to different Cyclins B isoforms

Different Cyclin B members are expressed before and after GVBD. In prophase oocytes, the main Cyclins correspond to Cyclin B2 and B5 isoforms and are associated with Cdk1 to form the pre-MPF. In response to progesterone, the synthesis of Cyclin B1 and B4 is initiated (Hochegger *et al.* 2001). The suppression of the synthesis of all Cyclins B isoforms abolishes the accumulation of endogenous Cdc6 after GVBD until the inactivation of the Mos/MAPK pathway. It will be therefore very interesting to study the accumulation of endogenous Cdc6 in oocytes injected with antisense targeting only one isoform of Cyclin B. If the elimination of B1 and B4 (GVBD Cyclins) delays Cdc6 accumulation while suppressing B2 and B5 (prophase Cyclins B) has not affect on Cdc6 accumulation, this would strongly suggest that only GVBD Cyclins are *in vivo* important for stabilizing Cdc6. This result would also provide some relevance for the presence of different Cyclins B isoforms during meiotic maturation.

Fig. 39: the sequence of *Xenopus* Cdc6

Xenopus Cdc6 sequence shows 3 consensus sites for CDKs (S74, S120 and S411), 2 motifs for MAPK (S45 and T88) and 2 others that can be phosphorylated by both CDK and MAPK (S54 and S108). In addition, a potential site of phosphorylation for Plk1 is present at S149. Finally, *Xenopus* Cdc6 contains sites controlling its turnover: as a PIP-box, F-box binding domain and two D-boxes.

iii. Different status of Cdk1-Cyclin B phosphorylation

At GVBD, it is known that the Cyclin B2 sustains a shift up in its electrophoretic migration that correlates with the activation of Cdk1. Whether the phosphorylation of Cyclin B can affect its interaction with Cdc6 could be explored. This hypothesis can be addressed by inactivating Cdk1 right after GVBD independently of Cyclin B degradation in order to generate some inactive pre-MPF molecules related to those present in prophase: Cyclin B associated with Cdk1 that is phosphorylated at T161, T14 and Y15. The injection of $p21^{Cip1}$ at GVBD inhibits Cdk1 and promotes its rephosphorylation at Y15. Under this condition, the Cyclins B are not degraded but are dephosphorylated (Frank-Vaillant *et al.* 2001). These Cdk1-Cyclin B complexes are therefore very similar to those present in prophase, despite the identity of the associated Cyclin B isoforms (B1, B2, B4, B5). If the interaction between Cdc6 and Cdk1-Cyclins B, and therefore Cdc6 stabilization, depends on the phosphorylation status of the MPF, recombinant Cdc6 should not be stable when co-injected with $p21^{Cip1}$ at GVBD.

iv. Localization of Cdc6 and Cdk1-Cyclin B complexes

Cyclin B2 is pulled down together with GST-Cdc6 in prophase-diluted extracts. This experiment demonstrates that Cyclin B and ectopic Cdc6 have the ability to interact but it does not allow to address the potential regulation exerted by intracellular compartments since in these extracts the intracellular organization is lost. My preliminary data indicate that ectopic Cdc6 is mainly localized within the nucleus in prophase while the pre-MPF is known to be localized in both the nucleus and the cytoplasm. It is therefore possible that due to these distinct localizations, only a part of Cdc6 interacts with Cyclin B. This different localization of Cdc6 and Cdk1-Cyclin B could account for the degradation of ectopic Cdc6 around the time of GVBD. The nuclear localization of Cdc6 would protect it from the degradation. At GVBD, Cdc6 becomes entirely cytoplasmic and could be fully accessible to the degradation mechanism. Therefore, one simple experiment to address this issue would be to analyse the degradation of ectopic Cdc6 in enucleated oocytes during meiosis resumption.

d. High molecular weight complexes containing both MPF and Cdc6

Only 50 % of the Cyclin B is degraded at MI exit. Hence, a residual Cdk1 activity persists during MI-MII transition. Cdk1 activity is required for the polyadenylation of Erp1 mRNA, which promotes its translation, independently of the Mos/MAPK pathway (Tung *et al.* 2007). Erp1 is essential for inhibiting APC/C and stabilizing newly synthesized Cyclin B, allowing entry into MII and then the

arrest at that stage (Ohe *et al.* 2007). From these observations, the presence of the residual activity of Cdk1 during MI-MII transition is required to enter in MII, promoting Erp1 translation (See chapter I-B.e.i.). The injection of 0.5 µM of WT-Cdc6 at GVBD induces full inactivation of Cdk1 and the entry in interphase. Hence, the absence of Cdc6 at GVBD is important to ensure the entry in MII.

Following the activation of MII-arrested oocytes, Erp1 gets fully degraded within 5 minutes and reaccumulates 40 minutes later (Schmidt *et al.* 2005). On the contrary, the Mos/MAPK pathway remains active for at least 30 minutes (Castro *et al.* 2001b). Following Erp1 degradation, the oocyte is under a biochemical status similar to anaphase I: the Mos/MAPK pathway is active and the activation of the APC/C leads to Cyclin B degradation. However, activated eggs do not perform an M- to M-phase transition but enter in interphase. This discrepancy between the exit from MI and the exit from MII can be due to the presence of the high levels of endogenous Cdc6 present in MII-arrested oocytes, which allow the full inactivation of Cdk1. In order to assess the potential role of endogenous Cdc6 in contributing to the complete inactivation of Cdk1 once the oocyte is released from the CSF arrest, it will be interesting to study the kinetic of Cdk1 inactivation in oocytes injected with antisense oligonucleotides against Cdc6.

Moreover, our gel filtration experiments demonstrate that endogenous Cdc6 is eluted together with Cdk1-Cyclin B complexes in high molecular weight fractions (H-MPF). Besides H-MPF, canonical Cyclin B-Cdk1 dimers (L-MPF) are also recovered in other fractions, as well as monomeric Cdk1. The high molecular weight pool of MPF was previously described in prophase-arrested oocytes (De Smedt *et al.* 2002), but its function has never been characterized. The formation of these MPF complexes depends neither on Cdc6 nor on the Mos/MAPK pathway since both Cdc6 and Mos are not expressed in prophase. Given the molecular weight of these complexes, they contain other molecules than Cyclin B, Cdk1 and Cdc6, but their components are not known. We showed that WT-Cdc6 injected at GVBD interacts only with the H-MPF. This pool of MPF could be an essential platform for the reactivation of MPF during the MI-MII transition. This exciting hypothesis can be investigated by performing several experiments. Since only 50 % of Cyclin B is degraded during MI-MII transition, we could analyse the elution profile of the remaining Cyclin B by fractionating oocytes collected 1 hour after GVBD on a gel filtration column. If the Cyclin B molecules included into H-MPF correspond to this stable pool, while the Cyclin B present in L-MPF is degraded, it will be a good indication that these two pools of MPF are differently regulated. Furthermore, it would suggest that the H-MPF could be specifically involved in keeping the residual level of Cdk1 activity in MI-MII and in the reactivation of Cdk1 for MII entry. This hypothesis as well as the physiological relevance of the specific association of Cdc6 with H-MPF, but not with L-MPF, deserves investigation.

ARTICLE II:
Fine-tuning of Cdc6 accumulation by Cdk1 and MAP kinase is essential for the completion of oocyte meiotic divisions

CONCLUSION and PERSPECTIVES

During my Ph.D., I investigated two main features of female meiosis: the prophase arrest and the establishment of DNA replication competence.

These two peculiarities of the female meiosis are directly connected to the high sexual dimorphism present in eukaryotic gametes. The female gamete, the oocyte, has to put aside many components falling into two categories: energetic nutrients and embryonic determinants. The first ones will be utilized by the embryo until its own genome is expressed, and its own metabolism as well as its ability to feed itself are established. The second ones that are often localized in specific area of the giant egg, will be inherited by embryonic cells and will orientate their fate and the organization of the whole body. Hence these stored components corresponds to mRNA, proteins, lipids, glucids, organelles, all of them required to sustain the first steps of the embryonic development until the moment the new formed organism will be autonomous. From that necessity, female meiosis is discontinuous and oocytes of all species of the animal kingdom arrest at prophase of the 1st meiotic division for a long time. Moreover, after prophase release, oocytes further arrest a second time to generate a time window required to reach a significant probability to meet sperm and proceed to fertilization. The arrest occurs at metaphase II in all vertebrates.

Both sperm and oocytes need to be haploid in order that fertilization restores a diploid genome that guarantees a successful life cycle. While in both sexes DNA replication has to be inhibited between the two meiotic divisions, the mechanisms to achieve this goal are different in females and males. Sperm uses the simplest strategy to avoid DNA replication: it downregulates the expression of pre-RC components during the last steps of its terminal differentiation (Eward *et al.* 2004). However, oocytes cannot do the same because they need to accumulate for the future embryo enough replicative components to ensure several rounds of embryonic divisions. By constituting this maternal inheritance, the oocytes acquire themselves the competence to replicate.

These “female duties” unfortunately has some costs. Firstly, the quantity of energy invested for the production of one gamete is much higher for females compared to males. Secondly, the long arrest in prophase I, which in human lasts for years, is thought to at the origin of the decrease of the quality of oocytes of elderly woman. Finally, the acquisition of DNA replication competence during meiosis, which is necessary for the embryo, includes a risk for the oocyte meiosis. Therefore, oocytes elaborated complex strategies to avoid the manifestation of that competence during meiotic divisions.

Since both male and female meiotic divisions certainly evolved from the mitotic cycle, with which they share many molecular players, it is therefore interesting to explore how the regulation of these common actors has been adapted in each three systems in order to produce the specific outcomes of each of them, mitotic cell cycle, female meiosis and male meiosis.

My first Ph.D. project reveals the involvement of Arpp19 during the prophase arrest of *Xenopus* oocytes. We have shown that Arpp19 is the long hidden substrate of PKA. Arpp19 is phosphorylated at S109, which is sufficient to arrest the oocytes in prophase. Moreover, S109 dephosphorylation is required for meiosis resumption. Surely, the natural continuation for this project is to elucidate how Arpp19 is blocking meiosis resumption in *Xenopus* oocytes, identifying which are its downstream targets. This discovery was especially unexpected since Arpp19 had been demonstrated a few years ago to be required for M-phase, including meiotic M-phase. Hence, the small protein Arpp19 behaves as the yin and the yang of the G_2/M transition. By virtue of the phosphorylation of two serines, S67 and S109, by two distinct kinases, Gwl and PKA, Arpp19 is converted from an inhibitor into an activator of M-phase. If this role is conserved in the somatic cell cycle, this places Arpp19 at a critical position for regulating proliferation, and as a potential hot spot responsible for proliferation disorders, such as cancer.

In all vertebrates the prophase arrest is maintained by high activity of PKA, suggesting that the role of the PKA/Arpp19 module should be conserved in this *phylum*, what deserves rapid investigation. The powerful genetic tools available in model organisms such as mouse and zebrafish should provide rapid insights about this question.

The release of the prophase block of invertebrate oocytes is ensured by a collection of various so-called "second messengers", such as Calcium, inositol-3-phosphate, etc. In some cases, as cnidarians and ctenarians, cAMP and PKA are essential for maintaining the prophase arrest, but in an opposite manner than what happens in vertebrates: prophase-arrested oocytes contain low levels of cAMP and low PKA activity. Both of them have to be increased for inducing the prophase release. A new emerging animal model for developmental biology, the small jelly fish *Clytia hemisphaerica*, is especially suited to study meiosis resumption and its control by cAMP and PKA (Takeda *et al.* 2006, Houliston *et al.* 2010). In *Clytia*, the light/dark stimulus stimulates photoreceptors expressed in somatic cells around the oocytes. These cells release a small peptide that reaches the oocytes, binds membrane receptors and increases cAMP levels and PKA activity (Takeda *et al.* 2006). It would be interesting to determine if the PKA/Arpp19 module mediates the cAMP-induced meiotic maturation in *Clytia*. If this is the case, the identification of Arpp19 effectors in vertebrates and invertebrates would be a great knowledge to understand how meiosis resumption can be regulated in an opposite manner by the same cAMP/PKA/Arpp19 module.

Finally, since the prophase arrest as many aspect of similarity with a mitotic G_2/M arrest, it will therefore interesting to investigate if the PKA/Arpp19 module can play any role during this transition in mitotic cells. Interestingly, cells activating a DNA damage checkpoint often arrest at the G_2/M transition and PKA site on Arpp19 is also a potential consensus motif for checkpoint mediator

kinases, Chk1/2. A potential role of Arpp19 in these checkpoint mechanisms deserves surely to be investigated.

In my second Ph.D. project, I investigated how is regulated the acquisition of DNA replication competence in *Xenopus* oocytes. We discovered that Cdc6 expression is tightly regulated during all the process of meiosis maturation. This regulation is absolutely essential after MI, a highly risky period where all components of the pre-RC machinery are present and functional, and where the oocyte has to avoid DNA replication. During the MI-MII transition we have shown that endogenous Cdc6 is post-translationally controlled by two opposite mechanisms. The Mos/MAPK pathway delays Cdc6 accumulation while it also facilitates the accumulation of Cyclin B, which is able to stabilize Cdc6. The overall output of this opposite regulation is that Cdc6 and Cyclin B are accumulated in a synchronous manner. We have also shown that a fine tuning of the Cdc6 dosage is essential for the successful sequence of the two meiotic divisions since its ectopic expression in prophase or in MI, alters Cdk1 activity.

Interestingly, the regulation of Cdc6 expression during meiosis resumption is well conserved in mouse and *Drosophila* oocytes: in both species, Cdc6 is not expressed in prophase oocytes, but the protein starts to accumulate during MI-MII transition, as in *Xenopus* (Lemaitre *et al.* 2004). In both mouse and *Xenopus*, and probably in all vertebrates, the Mos/MAPK pathway plays an important role in the inhibition of DNA replication during meiotic maturation (Colledge *et al.* 1994, Furuno *et al.* 1994, Hashimoto *et al.* 1994a, Verlhac *et al.* 1996a, Dupre *et al.* 2002). Since we have shown that the Mos/MAPK pathway kinase controls Cdc6 accumulation, contributing both to the regulation of Cdk1 reactivation and to DNA replication repression, it will be interesting to evaluate if Cdc6 regulation by the Mos/MAPK pathway is conserved in the other species. If it is the case, it suggests that vertebrate oocytes have developed two strategies to inhibit DNA replication during meiosis: ensuring the absence of Cdc6 in the first part of meiotic maturation (from the external stimulus to GVBD) and using the Mos/MAPK pathway during the second part. Interestingly, in unicellular eukaryotes as *S. pombe*, Cdc6 is not expressed during meiosis and therefore the haploid spores produced by meiosis are not competent to replicate DNA (Lemaitre *et al.* 2004). Therefore yeast has adopted a strategy to prevent DNA replication during meiosis similar to the one used by sperm or quiescent cells in higher eukaryotes. In contrast to *S. cerevisiae* and higher eukaryotes, the overexpression of Cdc6 during the mitotic cell cycle of *S. pombe* induces DNA re-replication, suggesting the absence of other mechanisms to control this deleterious process (Nishitani *et al.* 1995).

Besides its well-established role in DNA replication, this PhD work reveals that in a physiological situation, as *Xenopus* oocyte meiotic divisions, Cdc6 has the potential to inhibit Cdk1 by direct binding to accumulating Cyclin B. While in yeast, Cdc6 ability to regulate Cdk1 both through direct binding and by activating checkpoint mechanism is well established (Greenwood *et al.* 1998), mainly

the latter pathway has been characterized in higher eukaryotes (Oehlmann *et al.* 2004, Yoshida *et al.* 2010). Recently, the role of Cdc6 in modulating Cdk1 activation was investigated in *Xenopus* extracts (El Dika *et al.* 2014) and during the metaphase to anaphase transition in mammal cells (Yim *et al.* 2010). Unfortunately, Cdc6 plays many pleiotropic roles during mitosis, rendering difficult the interpretation of the consequences of its ablation. Despite this difficulty, the identification of the domains/sites in Cdc6 protein that are involved in each of these processes are required to define the importance of each of these roles during the cell cycle, and how they are coordinated.

Finally, the evidence that Cdc6 binds directly and specifically to MPF contained into high molecular weight complexes, but that it is able to inhibit the overall reactivation of MPF to drive the oocyte to the 2nd meiotic division opens the possibility of an unexpected role of these complexes in the control of the meiotic divisions. It will be therefore important to evaluate if these high molecular weight complexes are differentially regulated and play specific roles, compared to the canonical Cyclin B-Cdk1 dimer during meiosis as well as mitosis. This observation questions again the identity of MPF and re-opens the Pandora box: what is the MPF? In conclusion, the major lesson learned from my thesis work is that there is not end to questions.

REFERENCES

References

Abdel-Hafiz, H. A. and K. B. Horwitz (2014). "Post-translational modifications of the progesterone receptors." J. Steroid Biochem. Mol. Biol. **140**: 80-89.

Abrieu, A., L. Magnaghi-Jaulin, J. A. Kahana, M. Peter, A. Castro, S. Vigneron, T. Lorca, D. W. Cleveland and J. Labbe (2001). "Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint." Cell **106**(1): 83-93.

Adhikari, D., M. K. Diril, K. Busayavalasa, S. Risal, S. Nakagawa, R. Lindkvist, Y. Shen, V. Coppola, L. Tessarollo, N. R. Kudo, P. Kaldis and K. Liu (2014). "Mastl is required for timely activation of APC/C in meiosis I and Cdk1 reactivation in meiosis II." J. Cell Biol. **206**(7): 843-853.

Adhikari, D. and K. Liu (2013). "The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes." Mol. Cell. Endocrinol.

Alexandrow, M. G. and J. L. Hamlin (2004). "Cdc6 chromatin affinity is unaffected by serine-54 phosphorylation, S-phase progression, and overexpression of cyclin A." Mol Cell Biol **24**(4): 1614-1627.

Amiel, A., L. Leclere, L. Robert, S. Chevalier and E. Houliston (2009). "Conserved functions for Mos in eumetazoan oocyte maturation revealed by studies in a cnidarian." Curr. Biol. **19**(4): 305-311.

Anger, M., P. Stein and R. M. Schultz (2005). "CDC6 Requirement for Spindle Formation During Maturation of Mouse Oocytes." Biol. Reprod. **72**(1): 188-194.

Archambault, V., A. E. Ikui, B. J. Drapkin and F. R. Cross (2005). "Disruption of mechanisms that prevent rereplication triggers a DNA damage response." Mol Cell Biol **25**(15): 6707-6721.

Archambault, V., C. X. Li, A. J. Tackett, R. Wasch, B. T. Chait, M. P. Rout and F. R. Cross (2003). "Genetic and biochemical evaluation of the importance of Cdc6 in regulating mitotic exit." Mol. Biol. Cell **14**(11): 4592-4604.

Aressy, B. and B. Ducommun (2008). "Cell cycle control by the CDC25 phosphatases." Anticancer Agents Med Chem **8**(8): 818-824.

Arias, E. E. and J. C. Walter (2005). "Replication-dependent destruction of Cdt1 limits DNA replication to a single round per cell cycle in Xenopus egg extracts." Genes Dev **19**(1): 114-126.

Arias, E. E. and J. C. Walter (2006). "PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication." Natl. Cell Biol. **8**(1): 84-90.

Ayad, N. G., S. Rankin, M. Murakami, J. Jebanathirajah, S. Gygi and M. W. Kirschner (2003). "Tome-1, a trigger of mitotic entry, is degraded during G1 via the APC." Cell **113**(1): 101-113.

Aze, A., C. Fayet, L. Lapasset and A. M. Geneviere (2010). "Replication origins are already licensed in G1 arrested unfertilized sea urchin eggs." Dev. Biol. **340**(2): 557-570.

Bagowski, C. P., J. W. Myers and J. E. Ferrell, Jr. (2001). "The classical progesterone receptor associates with p42 MAPK and is involved in phosphatidylinositol 3-kinase signaling in *Xenopus* oocytes." J. Biol. Chem. **276**(40): 37708-37714.

Baldin, V. and B. Ducommun (1995). "Subcellular localisation of human wee1 kinase is regulated during the cell cycle." J. Cell Sci. **108 (Pt 6)**: 2425-2432.

Ballabeni, A., M. Melixetian, R. Zamponi, L. Masiero, F. Marinoni and K. Helin (2004). "Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis." EMBO J. **23**(15): 3122-3132.

Ballabeni, A., R. Zamponi, J. K. Moore, K. Helin and M. W. Kirschner (2013). "Geminin deploys multiple mechanisms to regulate Cdt1 before cell division thus ensuring the proper execution of DNA replication." Proc Natl Acad Sci U S A **110**(30): E2848-2853.

Baulieu, E. E., F. Godeau, M. Schorderet and S. Schorderet-Slatkine (1978). "Steroid-induced meiotic division in *Xenopus laevis* oocytes: surface and calcium." Nature **275**(5681): 593-598.

Belle, R., J. Boyer and R. Ozon (1984). "ATP-gamma-S (adenosine 5'-0(3-thiotriphosphate)) blocks progesterone-induced maturation of the Xenopus oocyte." J. Exp. Zool. **231**(1): 131-136.

Bhatt, R. R. and J. E. Ferrell, Jr. (1999). "The protein kinase p90 rsk as an essential mediator of cytostatic factor activity." Science **286**(5443): 1362-1365.

Blake-Hodek, K. A., B. C. Williams, Y. Zhao, P. V. Castilho, W. Chen, Y. Mao, T. M. Yamamoto and M. L. Goldberg (2012). "Determinants for Activation of the Atypical AGC Kinase Greatwall during M Phase Entry." Mol. Cell. Biol. **32**(8): 1337-1353.

Blow, J. J. and A. Dutta (2005). "Preventing re-replication of chromosomal DNA." Nat Rev Mol Cell Biol **6**(6): 476-486.

Boettcher, J. M., K. L. Hartman, D. T. Ladror, Z. Qi, W. S. Woods, J. M. George and C. M. Rienstra (2008). "Membrane-induced folding of the cAMP-regulated phosphoprotein endosulfine-alpha." Biochemistry **47**(47): 12357-12364.

Bollen, M., W. Peti, M. J. Ragusa and M. Beullens (2010). "The extended PP1 toolkit: designed to create specificity." Trends Biochem Sci **35**(8): 450-458.

Booher, R. N., P. S. Holman and A. Fattaey (1997). "Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity." J. Biol. Chem. **272**(35): 22300-22306.

Boonyaratanakornkit, V., Y. Bi, M. Rudd and D. P. Edwards (2008). "The role and mechanism of progesterone receptor activation of extra-nuclear signaling pathways in regulating gene transcription and cell cycle progression." Steroids **73**(9-10): 922-928.

Boutros, R., C. Dozier and B. Ducommun (2006). "The when and wheres of CDC25 phosphatases." Curr. Opin. Cell Biol. **18**(2): 185-191.

Boutros, R., V. Lobjois and B. Ducommun (2007). "CDC25 phosphatases in cancer cells: key players? Good targets?" Nat. Rev. Cancer **7**(7): 495-507.

Boyer, J., J. Asselin, R. Belle and R. Ozon (1986). "Progesterone and cAMP-dependent protein kinase regulate in vivo the level of phosphorylation of two proteins (Mr 20,000 and Mr 32,000) in Xenopus oocytes." Dev. Biol. **113**(2): 420-428.

Brunet, S., A. S. Maria, P. Guillaud, D. Dujardin, J. Z. Kubiak and B. Maro (1999). "Kinetochore fibers are not involved in the formation of the first meiotic spindle in mouse oocytes, but control the exit from the first meiotic M phase." J. Cell Biol. **146**(1): 1-12.

Bulavin, D. V., Z. N. Demidenko, C. Phillips, S. A. Moody and A. J. Fornace, Jr. (2003a). "Phosphorylation of Xenopus Cdc25C at Ser285 interferes with ability to activate a DNA damage replication checkpoint in pre-midblastula embryos." Cell Cycle **2**(3): 263-266.

Bulavin, D. V., Y. Higashimoto, Z. N. Demidenko, S. Meek, P. Graves, C. Phillips, H. Zhao, S. A. Moody, E. Appella, H. Piwnica-Worms and A. J. Fornace, Jr. (2003b). "Dual phosphorylation controls Cdc25 phosphatases and mitotic entry." Natl. Cell Biol. **5**(6): 545-551.

Burger, M. M., B. M. Bombik, B. M. Breckenridge and J. R. Sheppard (1972). "Growth control and cyclic alterations of cyclic AMP in the cell cycle." Nature: New biology **239**(93): 161-163.

Burgess, A., S. Vigneron, E. Brioudes, J. C. Labbe, T. Lorca and A. Castro (2010). "Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance." Proc Natl Acad Sci U S A.

Burrows, A. E., B. K. Sceurman, M. E. Kosinski, C. T. Richie, P. L. Sadler, J. M. Schumacher and A. Golden (2006). "The C. elegans Myt1 ortholog is required for the proper timing of oocyte maturation." Development **133**(4): 697-709.

Bzymek, M., N. H. Thayer, S. D. Oh, N. Kleckner and N. Hunter (2010). "Double Holliday junctions are intermediates of DNA break repair." Nature **464**(7290): 937-941.

Calzada, A., M. Sacristan, E. Sanchez and A. Bueno (2001). "Cdc6 cooperates with Sic1 and Hct1 to inactivate mitotic cyclin-dependent kinases." Nature **412**(6844): 355-358.

Castilho, P. V., B. C. Williams, S. Mochida, Y. Zhao and M. L. Goldberg (2009). "The M phase kinase Greatwall (Gwl) promotes inactivation of PP2A/B55delta, a phosphatase directed against CDK phosphosites." Mol. Biol. Cell **20**(22): 4777-4789.

Castro, A., M. Peter, T. Lorca and E. Mandart (2001a). "c-Mos and cyclin B/cdc2 connections during Xenopus oocyte maturation." Biology of the Cell (Paris) **93**(1-2): 15-25.

Castro, A., M. Peter, L. Magnaghi-Jaulin, S. Vigneron, S. Galas, T. Lorca and J. C. Labbe (2001b). "Cyclin B/cdc2 Induces c-Mos Stability by Direct Phosphorylation in Xenopus Oocytes." Mol. Biol. Cell **12**(9): 2660-2671.

Charlesworth, A., J. Welk and A. M. MacNicol (2000). "The temporal control of Wee1 mRNA translation during Xenopus oocyte maturation is regulated by cytoplasmic polyadenylation elements within the 3'-untranslated region." Dev. Biol. **227**(2): 706-719.

Chen, F., V. Archambault, A. Kar, P. Lio, P. P. D'Avino, R. Sinka, K. Lilley, E. D. Laue, P. Deak, L. Capalbo and D. M. Glover (2007). "Multiple protein phosphatases are required for mitosis in Drosophila." Curr. Biol. **17**(4): 293-303.

Chesnel, F., F. Bazile, A. Pascal and J. Z. Kubiak (2006). "Cyclin B dissociation from CDK1 precedes its degradation upon MPF inactivation in mitotic extracts of Xenopus laevis embryos." Cell Cycle **5**(15): 1687-1698.

Clay-Farrace, L., C. Pelizon, D. Santamaria, J. Pines and R. A. Laskey (2003). "Human replication protein Cdc6 prevents mitosis through a checkpoint mechanism that implicates Chk1." EMBO J. **22**(3): 704-712.

Clemenson, C. and M. C. Marsolier-Kergoat (2009). "DNA damage checkpoint inactivation: adaptation and recovery." DNA Repair (Amst) **8**(9): 1101-1109.

Clijsters, L. and R. Wolthuis (2014). "PIP-box-mediated degradation prohibits re-accumulation of Cdc6 during S phase." J. Cell Sci. **127**(Pt 6): 1336-1345.

Colledge, W. H., M. B. Carlton, G. B. Udy and M. J. Evans (1994). "Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs." Nature **370**(6484): 65-68.

Cook, J. G., C. H. Park, T. W. Burke, G. Leone, J. DeGregori, A. Engel and J. R. Nevins (2002). "Analysis of Cdc6 function in the assembly of mammalian prereplication complexes." Proc Natl Acad Sci U S A **99**(3): 1347-1352.

Cornwell, W. D., P. J. Kaminski and J. R. Jackson (2002). "Identification of Drosophila Myt1 kinase and its role in Golgi during mitosis." Cell. Signal. **14**(5): 467-476.

Coudreuse, D. and P. Nurse (2010). "Driving the cell cycle with a minimal CDK control network." Nature **468**(7327): 1074-1079.

Cundell, M. J., R. N. Bastos, T. Zhang, J. Holder, U. Gruneberg, B. Novak and F. A. Barr (2013). "The BEG (PP2A-B55/ENSA/Greatwall) Pathway Ensures Cytokinesis follows Chromosome Separation." Mol Cell.

Daar, I., N. Yew and G. F. Vande Woude (1993). "Inhibition of mos-induced oocyte maturation by protein kinase A." J. Cell Biol. **120**(5): 1197-1202.

Davidson, I. F., A. Li and J. J. Blow (2006). "Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision." Mol Cell **24**(3): 433-443.

De Smedt, V., R. Poulhe, X. Cayla, F. Dessauge, A. Karaiskou, C. Jessus and R. Ozon (2002). "Thr-161 phosphorylation of monomeric Cdc2. Regulation by protein phosphatase 2C in Xenopus oocytes." J. Biol. Chem. **277**(32): 28592-28600.

den Elzen, N. and J. Pines (2001). "Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase." J. Cell Biol. **153**(1): 121-136.

DePamphilis, M. L. (2005). "Cell cycle dependent regulation of the origin recognition complex." Cell Cycle **4**(1): 70-79.

Dillin, A. and J. Rine (1998). "Roles for ORC in M phase and S phase." Science **279**(5357): 1733-1737.

Dohadwala, M., E. Silva, F. L. Hall, R. T. Williams, D. A. Carbonarohall, A. C. Nairn, P. Greengard and N. Berndt (1994). "Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases." Proc. Natl. Acad. Sci. USA **91**(14): 6408-6412.

Doree, M., G. Peaucellier and A. Picard (1983). "Activity of the maturation-promoting factor and the extent of protein phosphorylation oscillate simultaneously during meiotic maturation of starfish oocytes." Dev. Biol. **99**(2): 489-501.

Drury, L. S., G. Perkins and J. F. Diffley (1997). "The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast." EMBO J. **16**(19): 5966-5976.

Du Pasquier, D., A. Dupre and C. Jessus (2011). "Unfertilized Xenopus eggs die by Bad-dependent apoptosis under the control of Cdk1 and JNK." PLoS One **6**(8): e23672.

Duckworth, B. C., J. S. Weaver and J. V. Ruderman (2002). "G2 arrest in *Xenopus* oocytes depends on phosphorylation of cdc25 by protein kinase A." Proc. Natl. Acad. Sci USA **99**(26): 16794-16799.

Dumont, J. N. (1972). "Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals." J. Morphol. **136**(2): 153-179.

Duncan, P. I., N. Pollet, C. Niehrs and E. A. Nigg (2001). "Cloning and characterization of Plx2 and Plx3, two additional Polo-like kinases from Xenopus laevis." Exp. Cell Res. **270**(1): 78-87.

Dunphy, W. G. and A. Kumagai (1991). "The cdc25 protein contains an intrinsic phosphatase activity." Cell **67**(1): 189-196.

Dupre, A., E. Buffin, C. Roustan, A. C. Nairn, C. Jessus and O. Haccard (2013). "The phosphorylation of ARPP19 by Greatwall renders the auto-amplification of MPF independently of PKA in Xenopus oocytes." J. Cell Sci. **126**(Pt 17): 3916-3926.

Dupre, A., E. M. Daldello, A. C. Nairn, C. Jessus and O. Haccard (2014). "Phosphorylation of ARPP19 by protein kinase A prevents meiosis resumption in Xenopus oocytes." Nat. Commun. **5**: 3318.

Dupre, A., O. Haccard and C. Jessus (2011). "Mos in the oocyte: how to use MAPK independently of growth factors and transcription to control meiotic divisions." J Signal Transduct **2011**: 350412.

Dupre, A., C. Jessus, R. Ozon and O. Haccard (2002). "Mos is not required for the initiation of meiotic maturation in *Xenopus* oocytes." EMBO J. **21**(15): 4026-4036.

Duursma, A. M. and R. Agami (2005). "CDK-dependent stabilization of Cdc6: linking growth and stress signals to activation of DNA replication." Cell Cycle **4**(12): 1725-1728.

Dyson, N. (1998). "The regulation of E2F by pRB-family proteins." Genes Dev **12**(15): 2245-2262.

Echalier, A., A. J. Hole, G. Lolli, J. A. Endicott and M. E. Noble (2014). "An inhibitor's-eye view of the ATP-binding site of CDKs in different regulatory states." ACS Chem Biol **9**(6): 1251-1256.

Eichenlaub-Ritter, U. and I. Boll (1989). "Nocodazole sensitivity, age-related aneuploidy, and alterations in the cell cycle during maturation of mouse oocytes." Cytogenet. Cell Genet. **52**(3-4): 170-176.

El Dika, M., K. Laskowska-Kaszub, M. Koryto, D. Dudka, C. Prigent, J. P. Tassan, M. Kloc, Z. Polanski, E. Borsuk and J. Z. Kubiak (2014). "CDC6 controls dynamics of the first embryonic M-phase entry and progression via CDK1 inhibition." Dev. Biol. **396**(1): 67-80.

El-Etr, M., S. Schorderet-Slatkine and E. E. Baulieu (1979). "Meiotic maturation in *Xenopus laevis* oocytes initiated by insulin." Science **205**(4413): 1397-1399.

Elia, A. E., L. C. Cantley and M. B. Yaffe (2003a). "Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates." Science **299**(5610): 1228-1231.

Elia, A. E., P. Rellos, L. F. Haire, J. W. Chao, F. J. Ivins, K. Hoepker, D. Mohammad, L. C. Cantley, S. J. Smerdon and M. B. Yaffe (2003b). "The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain." Cell **115**(1): 83-95.

Elsasser, S., Y. Chi, P. Yang and J. L. Campbell (1999). "Phosphorylation controls timing of Cdc6p destruction: A biochemical analysis." Mol. Biol. Cell **10**(10): 3263-3277.

Evans, T., E. T. Rosenthal, J. Youngblom, D. Distel and T. Hunt (1983). "Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division." Cell **33**(2): 389-396.

Evrin, C., P. Clarke, J. Zech, R. Lurz, J. Sun, S. Uhle, H. Li, B. Stillman and C. Speck (2009). "A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication." Proc Natl Acad Sci U S A **106**(48): 20240-20245.

Eward, K. L., E. C. Obermann, S. Shreeram, M. Loddo, T. Fanshawe, C. Williams, H. I. Jung, A. T. Prevost, J. J. Blow, K. Stoeber and G. H. Williams (2004). "DNA replication licensing in somatic and germ cells." J. Cell Sci. **117**(Pt 24): 5875-5886.

Fattaey, A. and R. N. Booher (1997). "Myt1: a Wee1-type kinase that phosphorylates Cdc2 on residue Thr14." Prog. Cell Cycle Res. **3**: 233-240.

Ferby, I., M. Blazquez, A. Palmer, R. Eritja and A. R. Nebreda (1999). "A novel p34(cdc2)-binding and activating protein that is necessary and sufficient to trigger G(2)/M progression in Xenopus oocytes." Genes Dev **13**(16): 2177-2189.

Fernandez, A., J. C. Cavadore, J. Demaille and N. Lamb (1995). "Implications for cAMP-dependent protein kinase in the maintenance of the interphase state." Prog. Cell Cycle Res. **1**: 241-253.

Fesquet, D., J. C. Labbe, J. Derancourt, J. P. Capony, S. Galas, F. Girard, T. Lorca, J. Shuttleworth, M. Doree and J. C. Cavadore (1993). "The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues." EMBO J. **12**(8): 3111-3121.

Finidori-lepicard, J., S. Schorderet-slatkine, J. Hanoune and E. E. Baulieu (1981). "Progesterone inhibits membrane bound adenylate cyclase in *Xenopus Laevis* oocytes." Nature **292**: 255-257.

Fisher, D. L., T. Brassac, S. Galas and M. Dorée (1999). "Dissociation of MAP kinase activation and MPF activation in hormone- stimulated maturation of Xenopus oocytes." Development **126**(20): 4537-4546.

Floyd, S., J. Pines and C. Lindon (2008). "APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase." Curr. Biol. **18**(21): 1649-1658.

Foulkes, J. G. and J. L. Maller (1982). "In vivo actions of protein phosphatase inhibitor-2 in *Xenopus* oocytes." FEBS Lett. **150**(1): 155-160.

Frank-Vaillant, M., O. Haccard, R. Ozon and C. Jessus (2001). "Interplay between Cdc2 kinase and the c-Mos/MAPK pathway between metaphase I and metaphase II in Xenopus oocytes." Dev. Biol. **231**(1): 279-288.

Frank-Vaillant, M., C. Jessus, R. Ozon, J. L. Maller and O. Haccard (1999). "Two distinct mechanisms control the accumulation of cyclin B1 and Mos in Xenopus oocytes in response to progesterone." Mol. Biol. Cell **10**(10): 3279-3288.

Frigola, J., D. Remus, A. Mehanna and J. F. Diffley (2013). "ATPase-dependent quality control of DNA replication origin licensing." Nature **495**(7441): 339-343.

Furnari, B., A. Blasina, M. N. Boddy, C. H. McGowan and P. Russell (1999). "Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1." Mol. Biol. Cell **10**(4): 833-845.

Furnari, B., N. Rhind and P. Russell (1997). "Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase." Science **277**(5331): 1495-1497.

Furuno, N., M. Nishizawa, K. Okazaki, H. Tanaka, J. Iwashita, N. Nakajo, Y. Ogawa and N. Sagata (1994). "Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes." Embo J. **13**(10): 2399-2410.

Gaffre, M., A. Martoriati, N. Belhachemi, J. P. Chambon, E. Houliston, C. Jessus and A. Karaiskou (2011). "A critical balance between Cyclin B synthesis and Myt1 activity controls meiosis entry in *Xenopus* oocytes." Development **138**(17): 3735-3744.

Gard, D. L. (1992). "Microtubule organization during maturation of Xenopus oocytes - assembly and rotation of the meiotic spindles." Develop. Biol. **151**: 516-530.

Gautier, J., T. Matsukawa, P. Nurse and J. Maller (1989). "Dephosphorylation and activation of *Xenopus* p34cdc2 protein kinase during the cell cycle." Nature **339**(6226): 626-629.

Gautier, J., J. Minshull, M. Lohka, M. Glotzer, T. Hunt and J. L. Maller (1990). "Cyclin is a component of maturation-promoting factor from Xenopus." Cell **60**(3): 487-494.

Gautier, J., C. Norbury, M. Lohka, P. Nurse and J. Maller (1988). "Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+." Cell **54**(3): 433-439.

Gautier, J., M. J. Solomon, R. N. Booher, J. F. Bazan and M. W. Kirschner (1991). "cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2." Cell **67**(1): 197-211.

Geley, S., E. Kramer, C. Gieffers, J. Gannon, J. M. Peters and T. Hunt (2001). "Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint." J. Cell Biol. **153**(1): 137-148.

Gerhart, J., M. Wu and M. Kirschner (1984). "Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs." J. Cell Biol. **98**(4): 1247-1255.

Gharbi-Ayachi, A., J. C. Labbe, A. Burgess, S. Vigneron, J. M. Strub, E. Brioudes, A. Van-Dorsselaer, A. Castro and T. Lorca (2010). "The substrate of Greatwall kinase, Arpp19, controls mitosis by inhibiting protein phosphatase 2A." Science **330**(6011): 1673-1677.

Ghosh, S., N. Paweletz and D. Schroeter (1998). "Cdc2-independent induction of premature mitosis by okadaic acid in HeLa cells." Exp. Cell Res. **242**(1): 1-9.

Girault, J. A., A. Horiuchi, E. L. Gustafson, N. L. Rosen and P. Greengard (1990). "Differential expression of ARPP-16 and ARPP-19, two highly related cAMP-regulated phosphoproteins, one of which is specifically associated with dopamine-innervated brain regions." J. Neurosci. **10**(4): 1124-1133.

Glotzer, M., A. W. Murray and M. W. Kirschner (1991). "Cyclin is degraded by the ubiquitin pathway." Nature **349**(6305): 132-138.

Godeau, J. F., S. Schorderet-Slatkine, P. Hubert and E. E. Baulieu (1978). "Induction of maturation in Xenopus laevis oocytes by a steroid linked to a polymer." Proc. Natl. Acad. Sci. USA **75**(5): 2353-2357.

Goldberg, M. L. (2010). "Greatwall kinase protects mitotic phosphosites from barbarian phosphatases." Proc Natl Acad Sci U S A **107**(28): 12409-12410.

Gorr, I. H., D. Boos and O. Stemmann (2005). "Mutual inhibition of separase and Cdk1 by two-step complex formation." Mol. Cell **19**(1): 135-141.

Goshima, G., M. Mayer, N. Zhang, N. Stuurman and R. D. Vale (2008). "Augmin: a protein complex required for centrosome-independent microtubule generation within the spindle." J. Cell Biol. **181**(3): 421-429.

Graves, P. R., C. M. Lovly, G. L. Uy and H. Piwnica-Worms (2001). "Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding." Oncogene **20**(15): 1839-1851.

Greenwood, E., H. Nishitani and P. Nurse (1998). "Cdc18p can block mitosis by two independent mechanisms." J. Cell Sci. **111 (Pt 20)**: 3101-3108.

Grimison, B., J. Liu, A. L. Lewellyn and J. L. Maller (2006). "Metaphase Arrest by Cyclin E-Cdk2 Requires the Spindle-Checkpoint Kinase Mps1." Curr. Biol. **16**(19): 1968-1973.

Gross, S. D., A. L. Lewellyn and J. L. Maller (2001). "A constitutively active form of the protein kinase p90Rsk1 is sufficient to trigger the G2/M transition in *Xenopus* oocytes." J. Biol. Chem. **276**(49): 46099-46103.

Gross, S. D., M. S. Schwab, F. E. Taieb, A. L. Lewellyn, Y. W. Qian and J. L. Maller (2000). "The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90(Rsk)." Curr. Biol. **10**(8): 430-438.

Guadagno, T. and J. Newport (1996). "Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity." Cell **84**(1): 73-82.

Gutierrez, G. J., A. Vogtlin, A. Castro, I. Ferby, G. Salvagiotto, Z. Ronai, T. Lorca and A. R. Nebreda (2006). "Meiotic regulation of the CDK activator RINGO/Speedy by ubiquitin-proteasome-mediated processing and degradation." Natl. Cell Biol. **8**(10): 1084-1094.

Haccard, O., A. Dupre, P. Liere, A. Pianos, B. Eychenne, C. Jessus and R. Ozon (2012). "Naturally occurring steroids in Xenopus oocyte during meiotic maturation. Unexpected presence and role of steroid sulfates." Mol. Cell. Endocrinol. **362**(1-2): 110-119.

Haccard, O. and C. Jessus (2006). "Redundant pathways for Cdc2 activation in *Xenopus* oocyte: either cyclin B or Mos synthesis." EMBO Rep. **7**(3): 321-325.

Haccard, O., A. Lewellyn, R. S. Hartley, E. Erikson and J. L. Maller (1995). "Induction of *Xenopus* oocyte meiotic maturation by MAP kinase." Dev. Biol. **168**(2): 677-682.

Haccard, O., B. Sarcevic, A. Lewellyn, R. Hartley, L. Roy, T. Izumi, E. Erikson and J. L. Maller (1993). "Induction of metaphase arrest in cleaving Xenopus embryos by MAP kinase." Science **262**(5137): 1262-1265.

Hached, K., S. Z. Xie, E. Buffin, D. Cladiere, C. Rachez, M. Sacras, P. K. Sorger and K. Wassmann (2011). "Mps1 at kinetochores is essential for female mouse meiosis I." Development **138**(11): 2261-2271.

Hampl, A. and J. J. Eppig (1995a). "Analysis of the mechanism(s) of metaphase I arrest in maturing mouse oocytes." Development **121**(4): 925-933.

Hampl, A. and J. J. Eppig (1995b). "Translational regulation of the gradual increase in histone H1 kinase activity in maturing mouse oocytes." Mol. Reprod. Dev. **40**: 9-15.

Han, S. J., R. Chen, M. P. Paronetto and M. Conti (2005). "Wee1B Is an Oocyte-Specific Kinase Involved in the Control of Meiotic Arrest in the Mouse." Curr. Biol. **15**(18): 1670-1676.

Hansen, D. V., J. J. Tung and P. K. Jackson (2006). "CaMKII and polo-like kinase 1 sequentially phosphorylate the cytostatic factor Emi2/XErp1 to trigger its destruction and meiotic exit." Proc. Natl. Acad. Sci. USA **103**(3): 608-613.

Hara, M., Y. Abe, T. Tanaka, T. Yamamoto, E. Okumura and T. Kishimoto (2012). "Greatwall kinase and cyclin B-Cdk1 are both critical constituents of M-phase-promoting factor." Nat. Commun. **3**: 1059.

Harper, J. W., J. L. Burton and M. J. Solomon (2002). "The anaphase-promoting complex: it's not just for mitosis any more." Genes Dev **16**(17): 2179-2206.

Hashimoto, N., N. Watanabe, Y. Furuta, H. Tamemoto, N. Sagata, M. Yokoyama, K. Okazaki, M. Nagayoshi, N. Takeda, Y. Ikawa and S. Aizawa (1994). "Parthenogenetic activation of oocytes in c-mos-deficient mice." Nature **370**(6484): 68-71.

Hassold, T. and P. Hunt (2009). "Maternal age and chromosomally abnormal pregnancies: what we know and what we wish we knew." Curr. Opin. Pediatr. **21**(6): 703-708.

Hauf, S., E. Roitinger, B. Koch, C. M. Dittrich, K. Mechtler and J. M. Peters (2005). "Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2." PLoS Biol. **3**(3): e69.

Heald, R., M. McLoughlin and F. McKeon (1993). "Human wee-1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase." Cell **74**: 463-474.

Helps, N. R., X. Luo, H. M. Barker and P. T. Cohen (2000). "NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1." Biochem. J. **349**(Pt 2): 509-518.

Hermand, D. and P. Nurse (2007). "Cdc18 enforces long-term maintenance of the S phase checkpoint by anchoring the Rad3-Rad26 complex to chromatin." Mol Cell **26**(4): 553-563.

Hill, R. S. and H. C. Macgregor (1980). "The development of lampbrush chromosome-type transcription in the early diplotene oocytes of Xenopus laevis: an electron-microscope analysis." J. Cell Sci. **44**: 87-101.

Hochegger, H., A. Klotzbucher, J. Kirk, M. Howell, K. le Guellec, K. Fletcher, T. Duncan, M. Sohail and T. Hunt (2001). "New B-type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation." Development **128**(19): 3795-3807.

Hoffmann, I., P. R. Clarke, M. J. Marcote, E. Karsenti and G. Draetta (1993). "Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis." EMBO J. **12**(1): 53-63.

Hoffmann, S., C. Tsurumi, J. Z. Kubiak and Z. Polanski (2006). "Germinal vesicle material drives meiotic cell cycle of mouse oocyte through the 3'UTR-dependent control of cyclin B1 synthesis." Dev. Biol. **292**(1): 46-54.

Hofmann, J. F. and D. Beach (1994). "cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis." EMBO J. **13**(2): 425-434.

Holland, A. J. and S. S. Taylor (2006). "Cyclin-B1-mediated inhibition of excess separase is required for timely chromosome disjunction." J. Cell Sci. **119**(Pt 16): 3325-3336.

Homer, H. A., A. McDougall, M. Levasseur, A. P. Murdoch and M. Herbert (2005). "Mad2 is required for inhibiting securin and cyclin B degradation following spindle depolymerisation in meiosis I mouse oocytes." Reproduction (Cambridge, England) **130**(6): 829-843.

Houliston, E., T. Momose and M. Manuel (2010). "Clytia hemisphaerica: a jellyfish cousin joins the laboratory." Trends Genet. **26**(4): 159-167.

Hoyt, M. A., L. Totis and B. T. Roberts (1991). "*S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function." Cell **66**: 507-517.

Huchon, D. and R. Ozon (1979). "Effects of 3-isobutyl-methylxanthine (IBMX) and of cholera toxin on maturation induced by the injection of maturation promoting factor (MPF) in oocyte of Xenopus laevis." Biol. Cell **35**: 15-20.

Huchon, D., N. Crozet, N. Cantenot and R. Ozon (1981a). "Germinal vesicle breakdown in the *Xenopus laevis* oocyte: description of a transient microtubular structure." Reprod. Nutr. Dev. **21**(1): 135-148.

Huchon, D., R. Ozon and J. G. Demaille (1981b). "Protein phosphatase-1 is involved in *Xenopus* oocyte maturation." Nature **294**(5839): 358-359.

Huchon, D., R. Ozon, E. H. Fischer and J. G. Demaille (1981c). "The pure inhibitor of cAMP-dependent protein kinase initiates Xenopus laevis meiotic maturation. A 4-step scheme for meiotic maturation." Mol. Cell. Endocrinol. **22**(2): 211-222.

Huchon, D., H. Rime, C. Jessus and R. Ozon (1993). "Control of metaphase I formation in Xenopus oocyte: effects of an indestructible cyclin B and of protein synthesis." Biol. Cell **77**(2): 133-141.

Hutchins, J. R., D. Dikovskaya and P. R. Clarke (2003). "Regulation of Cdc2/cyclin B activation in Xenopus egg extracts via inhibitory phosphorylation of Cdc25C phosphatase by Ca(2+)/calmodulin-dependent protein [corrected] kinase II." Mol. Biol. Cell **14**(10): 4003-4014.

Illenye, S. and N. H. Heintz (2004). "Functional analysis of bacterial artificial chromosomes in mammalian cells: mouse Cdc6 is associated with the mitotic spindle apparatus." Genomics **83**(1): 66-75.

Inoue, D., M. Ohe, Y. Kanemori, T. Nobui and N. Sagata (2007). "A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of Xenopus laevis eggs." Nature **446**(7139): 1100-1104.

Ishikawa, K., Y. Hanaoka, Y. Kondo and K. Imai (1977). "Primary action of steroid hormone at the surface of amphibian oocyte in the induction of germinal vesicle breakdown." Mol. Cell. Endocrinol. **9**(1): 91-100.

Iwabuchi, M., K. Ohsumi, T. M. Yamamoto, W. Sawada and T. Kishimoto (2000). "Residual Cdc2 activity remaining at meiosis I exit is essential for meiotic M-M transition in Xenopus oocyte extracts." EMBO J. **19**(17): 4513-4523.

Iwashita, J., Y. Hayano and N. Sagata (1998). "Essential role of germinal vesicle material in the meiotic cell cycle of Xenopus oocytes." Proc. Natl. Acad. Sci. USA **95**(8): 4392-4397.

Izumi, T. and J. L. Maller (1993). "Elimination of Cdc2 Phosphorylation Sites in the Cdc25 Phosphatase Blocks Initiation of M-Phase." Mol. Biol. Cell **4**(12): 1337-1350.

Izumi, T. and J. L. Maller (1995). "Phosphorylation and activation of the *Xenopus* Cdc25 phosphatase in the absence of Cdc2 and Cdk2 kinase activity." Mol. Biol. Cell **6**(2): 215-226.

Izumi, T., D. H. Walker and J. L. Maller (1992). "Periodic changes in phosphorylation of the *Xenopus* Cdc25 phosphatase regulate its activity." Mol. Biology of the Cell **3**: 927-939.

Jackman, M., C. Lindon, E. A. Nigg and J. Pines (2003). "Active cyclin B1-Cdk1 first appears on centrosomes in prophase." Natl. Cell Biol. **5**(2): 143-148.

Jacobelli, S., J. Hanocq, E. Baltus and J. Brachet (1974). "Hormone-induced maturation of Xenopus laevis oocytes: effects of different steroids and study of the properties of a progesterone receptor." Differentiation **2**: 129-113.

Jacobsen, B. M. and K. B. Horwitz (2012). "Progesterone receptors, their isoforms and progesterone regulated transcription." Mol. Cell. Endocrinol. **357**(1-2): 18-29.

Jeffrey, P. D., A. A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Massague and N. P. Pavletich (1995). "Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex." Nature **376**(6538): 313-320.

Jessus, C. and D. Beach (1992). "Oscillation of MPF is accompanied by periodic association between cdc25 and cdc2-cyclin B." Cell **68**: 323-332.

Jiang, W., N. J. Wells and T. Hunter (1999). "Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6." Proc Natl Acad Sci U S A **96**(11): 6193-6198.

Jin, Z., E. M. Homola, P. Goldbach, Y. Choi, J. A. Brill and S. D. Campbell (2005). "Drosophila Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis." Development **132**(18): 4075-4085.

Josefsberg Ben-Yehoshua, L., A. L. Lewellyn, P. Thomas and J. L. Maller (2007). "The role of Xenopus membrane progesterone receptor beta in mediating the effect of progesterone on oocyte maturation." Mol. Endocrinol. **21**(3): 664-673.

Juanes, M. A., R. Khoueiry, T. Kupka, A. Castro, I. Mudrak, E. Ogris, T. Lorca and S. Piatti (2013). "Budding Yeast Greatwall and Endosulfines Control Activity and Spatial Regulation of PP2A(Cdc55) for Timely Mitotic Progression." PLoS Genet. **9**(7): e1003575.

Kajiura-Kobayashi, H., N. Yoshida, N. Sagata, M. Yamashita and Y. Nagahama (2000). "The Mos/MAPK pathway is involved in metaphase II arrest as a cytostatic factor but is neither necessary nor sufficient for initiating oocyte maturation in goldfish." Dev Genes Evol **210**(8-9): 416-425.

Karaiskou, A., X. Cayla, O. Haccard, C. Jessus and R. Ozon (1998). "MPF amplification in *Xenopus* oocyte extracts depends on a two-step activation of cdc25 phosphatase." Exp. Cell Res. **244**(2): 491-500.

Karaiskou, A., C. Jessus, T. Brassac and R. Ozon (1999). "Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification." J. Cell Sci. **112 (Pt 21)**(Pt 21): 3747-3756.

Karaiskou, A., A. C. Lepretre, G. Pahlavan, D. Du Pasquier, R. Ozon and C. Jessus (2004). "Polo-like kinase confers MPF autoamplification competence to growing Xenopus oocytes." Development **131**(7): 1543-1552.

Karlsson-Rosenthal, C. and J. B. Millar (2006). "Cdc25: mechanisms of checkpoint inhibition and recovery." Trends Cell Biol.

Katayama, K., N. Fujita and T. Tsuruo (2005). "Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle progression at G2/M transition." Mol Cell Biol **25**(13): 5725-5737.

Katsu, Y., M. Yamashita, H. Kajiura and Y. Nagahama (1993). "Behavior of the components of maturation-promoting factor, cdc2 kinase and cyclin B, during oocyte maturation of goldfish." Dev. Biol. **160**(1): 99-107.

Kelly, T. J., G. S. Martin, S. L. Forsburg, R. J. Stephen, A. Russo and P. Nurse (1993). "The fission yeast cdc18+ gene product couples S phase to START and mitosis." Cell **74**(2): 371-382.

Kelm, O., M. Wind, W. D. Lehmann and E. A. Nigg (2002). "Cell cycle-regulated phosphorylation of the Xenopus polo-like kinase Plx1." J. Biol. Chem. **277**(28): 25247-25256.

King, R. W., J.-M. Peters, S. Tugendreich, M. Rolfe, P. Hieter and M. W. Kirschner (1995). "A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B." Cell **81**(1): 279-288.

Kinoshita, N., H. Ohkura and M. Yanagida (1990). "Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle." Cell **63**: 405-415.

Kitajima, T. S., S. A. Kawashima and Y. Watanabe (2004). "The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis." Nature **427**(6974): 510-517.

Kitajima, T. S., T. Sakuno, K. Ishiguro, S. Iemura, T. Natsume, S. A. Kawashima and Y. Watanabe (2006). "Shugoshin collaborates with protein phosphatase 2A to protect cohesin." Nature **441**(7089): 46-52.

Kobayashi, H., J. Minshull, C. Ford, R. Golsteyn, R. Poon and T. Hunt (1991). "On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation in *Xenopus* laevis." J. Cell Biol. **114**(4): 755-765.

Kobayashi, H., E. Stewart, R. Poon, J. P. Adamczewski, J. Gannon and T. Hunt (1992). "Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits." Mol. Biol. Cell **3**(11): 1279-1294.

Kornbluth, S., B. Sebastian, T. Hunter and J. Newport (1994). "Membrane localization of the kinase which phosphorylates $p34^{cdc2}$ on threonine 14." Mol. Biology of the Cell. **5**(3): 273-282.

Krek, W. and E. A. Nigg (1992). "Cell cycle regulation of vertebrate p34cdc2 activity: identification of Thr161 as an essential in vivo phosphorylation site." New Biol **4**(4): 323-329.

Kumagai, A. and W. G. Dunphy (1992). "Regulation of the Cdc25 protein during the cell cycle in *Xenopus* extracts." Cell **70**: 139-151.

Kumagai, A. and W. G. Dunphy (1996). "Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts." Science **273**(5280): 1377-1380.

Kumagai, A. and W. G. Dunphy (1999). "Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25." Genes Dev **13**(9): 1067-1072.

Kumagai, A., Z. Guo, K. H. Emami, S. X. Wang and W. G. Dunphy (1998a). "The Xenopus Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts." J. Cell Biol. **142**(6): 1559-1569.

Kumagai, A., P. S. Yakowec and W. G. Dunphy (1998b). "14-3-3 Proteins Act As Negative Regulators Of the Inducer Cdc25 In Xenopus Egg Extracts." Mol. Biol. Cell **9**(2): 345-354.

Kurokawa, K. and J. Kato (1998). "Cyclic AMP delays G2 progression and prevents efficient accumulation of cyclin B1 proteins in mouse macrophage cells." Cell Struct. Funct. **23**(6): 357-365.

Kwon, Y. G., S. Y. Lee, Y. W. Choi, P. Greengard and A. C. Nairn (1997). "Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase." Proc. Natl. Acad. Sci. USA **94**(6): 2168-2173.

Labandera, A. M., A. R. Vahab, S. Chaudhuri, D. Kerk and G. B. Moorhead (2015). "The mitotic PP2A regulator ENSA/ARPP-19 is remarkably conserved across plants and most eukaryotes." Biochem. Biophys. Res. Commun. **458**(4): 739-744.

Labib, K., J. F. Diffley and S. E. Kearsey (1999). "G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus." Natl. Cell Biol. **1**(7): 415-422.

Lamb, J. C., J. C. Cavadore, J. C. Labbe, R. Maurer and A. Fernandez (1991). "Inhibition of cAMP-dependent protein kinase plays a key role in the induction of meiosis and nuclear envelope breakdown in mammalian cells." EMBO J. **10**: 1523-1533.

Langerak, P. and P. Russell (2011). "Regulatory networks integrating cell cycle control with DNA damage checkpoints and double-strand break repair." Philos. Trans. R. Soc. Lond. B. Biol. Sci. **366**(1584): 3562-3571.

Lara-Gonzalez, P., F. G. Westhorpe and S. S. Taylor (2012). "The spindle assembly checkpoint." Curr. Biol. **22**(22): R966-980.

Larochelle, S., K. A. Merrick, M. E. Terret, L. Wohlbold, N. M. Barboza, C. Zhang, K. M. Shokat, P. V. Jallepalli and R. P. Fisher (2007). "Requirements for Cdk7 in the assembly of Cdk1/cyclin B and activation of Cdk2 revealed by chemical genetics in human cells." Mol Cell **25**(6): 839-850.

Lau, E., C. Zhu, R. T. Abraham and W. Jiang (2006). "The functional role of Cdc6 in S-G2/M in mammalian cells." EMBO Rep.

Ledan, E., Z. Polanski, M. E. Terret and B. Maro (2001). "Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation." Dev. Biol. **232**(2): 400-413.

Lee, J., A. Kumagai and W. G. Dunphy (2001). "Positive regulation of wee1 by chk1 and 14-3-3 proteins." Mol. Biol. Cell **12**(3): 551-563.

Lee, M. S., S. Ogg, M. Xu, L. L. Parker, D. J. Donoghue, J. L. Maller and H. Piwnica-Worms (1992). "cdc25+ encodes a protein phosphatase that dephosphorylates p34cdc2." Mol. Biol. Cell **3**(1): 73-84.

Leise, W., 3rd and P. R. Mueller (2002). "Multiple Cdk1 inhibitory kinases regulate the cell cycle during development." Dev. Biol. **249**(1): 156-173.

Leland, S., P. Nagarajan, A. Polyzos, S. Thomas, G. Samaan, R. Donnell, F. Marchetti and S. Venkatachalam (2009). "Heterozygosity for a Bub1 mutation causes female-specific germ cell aneuploidy in mice." Proc Natl Acad Sci U S A **106**(31): 12776-12781.

Lemaitre, J. M., S. Bocquet and M. Mechali (2002). "Competence to replicate in the unfertilized egg is conferred by Cdc6 during meiotic maturation." Nature **419**(6908): 718-722.

Lemaitre, J. M., S. Bocquet, M. E. Terret, M. Namdar, O. Ait-Ahmed, S. Kearsey, M. H. Verlhac and M. Mechali (2004). "The regulation of competence to replicate in meiosis by Cdc6 is conserved during evolution." Mol. Reprod. Dev. **69**(1): 94-100.

Lenormand, J. L., R. W. Dellinger, K. E. Knudsen, S. Subramani and D. J. Donoghue (1999). "Speedy: a novel cell cycle regulator of the G2/M transition." EMBO J. **18**(7): 1869-1877.

Lewis, C. W., R. G. Taylor, P. M. Kubara, K. Marshall, L. Meijer and R. M. Golsteyn (2013). "A western blot assay to measure cyclin dependent kinase activity in cells or in vitro without the use of radioisotopes." FEBS Lett. **587**(18): 3089-3095.

Li, A. and J. J. Blow (2005). "Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus." EMBO J. **24**(2): 395-404.

Li, J. L., Y. C. Cai, X. H. Liu and L. J. Xian (2006). "Norcantharidin inhibits DNA replication and induces apoptosis with the cleavage of initiation protein Cdc6 in HL-60 cells." Anticancer Drugs **17**(3): 307-314.

Li, M., X. Fang, Z. Wei, J. P. York and P. Zhang (2009). "Loss of spindle assembly checkpoint-mediated inhibition of Cdc20 promotes tumorigenesis in mice." J. Cell Biol. **185**(6): 983-994.

Li, R. and A. W. Murray (1991). "Feedback control of mitosis in budding yeast." Cell **66**: 519-531.

Lichten, M. and A. S. Goldman (1995). "Meiotic recombination hotspots." Annu. Rev. Genet. **29**: 423-444.

Lindon, C. and J. Pines (2004). "Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells." J. Cell Biol. **164**(2): 233-241.

Lindqvist, A., H. Kallstrom, A. Lundgren, E. Barsoum and C. K. Rosenthal (2005). "Cdc25B cooperates with Cdc25A to induce mitosis but has a unique role in activating cyclin B1-Cdk1 at the centrosome." J. Cell Biol. **171**(1): 35-45.

Liu, D., M. Vleugel, C. B. Backer, T. Hori, T. Fukagawa, I. M. Cheeseman and M. A. Lampson (2010). "Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase." J. Cell Biol. **188**(6): 809-820.

Liu, F., C. Rothblum-Oviatt, C. E. Ryan and H. Piwnica-Worms (1999). "Overproduction of human Myt1 kinase induces a G2 cell cycle delay by interfering with the intracellular trafficking of Cdc2-cyclin B1 complexes." Mol. Cell. Biol. **19**(7): 5113-5123.

Liu, J. and J. L. Maller (2005a). "Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CaMK II to release metaphase arrest by cytostatic factor." Curr. Biol. **15**(16): 1458-1468.

Liu, X. S., C. Ma, A. W. Hamam and X. J. Liu (2005b). "Transcription-dependent and transcription-independent functions of the classical progesterone receptor in Xenopus ovaries." Dev. Biol. **283**(1): 180-190.

Lohka, M. J., M. K. Hayes and J. L. Maller (1988). "Purification of maturation-promoting factor, an intracellular regulator of early mitotic events." Proc Natl Acad Sci U S A **85**(9): 3009-3013.

Lohka, M. J. and J. L. Maller (1985). "Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts." J. Cell Biol. **101**(2): 518-523.

Lopez-Girona, A., J. Kanoh and P. Russell (2001). "Nuclear exclusion of Cdc25 is not required for the DNA damage checkpoint in fission yeast." Curr. Biol. **11**(1): 50-54.

Lorca, T. and A. Castro (2013). "Deciphering the New Role of the Greatwall/PP2A Pathway in Cell Cycle Control." Genes & Cancer **in press**.

Lorca, T., A. Castro, A. M. Martinez, S. Vigneron, N. Morin, S. Sigrist, C. Lehner, M. Doree and J. C. Labbe (1998). "Fizzy is required for activation of the APC/cyclosome in Xenopus egg extracts." EMBO J. **17**(13): 3565-3575.

Lorca, T., F. H. Cruzalegui, D. Fesquet, J. C. Cavadore, J. Mery, A. Means and M. Doree (1993). "Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs." Nature **366**(6452): 270-273.

Lorca, T., S. Galas, D. Fresquet, A. Devault, J. C. Cavadore and M. Doree (1991). "Degradation of the proto-oncogene product p39mos is not necessary for cyclin proteolysis and exit from meiotic metaphase : requirement for a Ca2+-calmodulin dependent event." EMBO J. **10**: 2087-2093.

Luo, X., N. Talarek and C. De Virgilio (2011). "Initiation of the yeast G0 program requires Igo1 and Igo2, which antagonize activation of decapping of specific nutrient-regulated mRNAs." RNA Biology **8**(1): 14-17.

Lutzmann, M., D. Maiorano and M. Mechali (2006). "A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in Xenopus." EMBO J. **25**(24): 5764-5774.

Mailand, N. and J. F. Diffley (2005). "CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis." Cell **122**(6): 915-926.

Mailand, N., A. V. Podtelejnikov, A. Groth, M. Mann, J. Bartek and J. Lukas (2002). "Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability." EMBO J. **21**(21): 5911-5920.

Maiorano, D., L. Krasinska, M. Lutzmann and M. Mechali (2005). "Recombinant Cdt1 induces rereplication of G2 nuclei in Xenopus egg extracts." Curr. Biol. **15**(2): 146-153.

Maller, J. L. (2001). "The elusive progesterone receptor in Xenopus oocytes." Proc. Natl. Acad. Sci. USA **98**(1): 8-10.

Maller, J. L. and J. W. Koontz (1981). "A study of the induction of cell division in amphibian oocytes by insulin." Dev. Biol. **85**(2): 309-316.

Maller, J. L. and E. G. Krebs (1977). "Progesterone-stimulated meiotic cell division in Xenopus oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase." J. Biol. Chem. **252**(5): 1712-1718.

Maller, J. L. and E. G. Krebs (1980). "Regulation of oocyte maturation." Curr. Top. Cell. Regul. **16**: 271-311.

Malumbres, M. (2014). "Cyclin-dependent kinases." Genome Biol **15**(6): 122.

Margolis, S. S., S. Walsh, D. C. Weiser, M. Yoshida, S. Shenolikar and S. Kornbluth (2003). "PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation." EMBO J. **22**(21): 5734-5745.

Margolis, S. S., J. A. Perry, C. M. Forester, L. K. Nutt, Y. Guo, M. J. Jardim, M. J. Thomenius, C. D. Freel, R. Darbandi, J. H. Ahn, J. D. Arroyo, X. F. Wang, S. Shenolikar, A. C. Nairn, W. G. Dunphy, W. C. Hahn, D. M. Virshup and S. Kornbluth (2006a). "Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis." Cell **127**(4): 759-773.

Margolis, S. S., J. A. Perry, D. H. Weitzel, C. D. Freel, M. Yoshida, T. A. Haystead and S. Kornbluth (2006b). "A role for PP1 in the Cdc2/Cyclin B-mediated positive feedback activation of Cdc25." Mol. Biol. Cell **17**(4): 1779-1789.

Margottin-Goguet, F., J. Y. Hsu, A. Loktev, H. M. Hsieh, J. D. Reimann and P. K. Jackson (2003). "Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase." Dev. Cell **4**(6): 813-826.

Martinez, J. S., H. Hall, M. D. Bartolowits and M. C. Hall (2012). "Acm1 contributes to nuclear positioning by inhibiting Cdh1-substrate interactions." Cell Cycle **11**(2): 384-394.

Martinez, J. S., D. E. Jeong, E. Choi, B. M. Billings and M. C. Hall (2006). "Acm1 is a negative regulator of the CDH1-dependent anaphase-promoting complex/cyclosome in budding yeast." Mol Cell Biol **26**(24): 9162-9176.

Masui, Y. and C. L. Markert (1971). "Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes." J. Exp. Zool. **177**(2): 129-145.

Matten, W., I. Daar and G. F. V. Woude (1994). "Protein-Kinase a Acts at Multiple Points to Inhibit Xenopus-Oocyte Maturation." Mol. Cell. Biol. **14**(7): 4419-4426.

Matthews, L. M. and J. P. Evans (2014). "alpha-endosulfine (ENSA) regulates exit from prophase I arrest in mouse oocytes." Cell Cycle **13**(10): 1639-1649.

McCoy, F., R. Darbandi, S. I. Chen, L. Eckard, K. Dodd, K. Jones, A. J. Baucum, 2nd, J. A. Gibbons, S. H. Lin, R. J. Colbran and L. K. Nutt (2013). "Metabolic regulation of CaMKII protein and caspases in Xenopus laevis egg extracts." J. Biol. Chem. **288**(13): 8838-8848.

McGowan, C. H. and P. Russell (1995). "Cell cycle regulation of human WEE1." EMBO J. **14**(10): 2166-2175.

McGuinness, B. E., M. Anger, A. Kouznetsova, A. M. Gil-Bernabe, W. Helmhart, N. R. Kudo, A. Wuensche, S. Taylor, C. Hoog, B. Novak and K. Nasmyth (2009). "Regulation of APC/C activity in oocytes by a Bub1-dependent spindle assembly checkpoint." Curr. Biol. **19**(5): 369-380.

Meadows, J. C., L. A. Shepperd, V. Vanoosthuyse, T. C. Lancaster, A. M. Sochaj, G. J. Buttrick, K. G. Hardwick and J. B. Millar (2011). "Spindle checkpoint silencing requires association of PP1 to both Spc7 and kinesin-8 motors." Dev. Cell **20**(6): 739-750.

Mehlmann, L. M. (2005). "Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation." Reproduction (Cambridge, England) **130**(6): 791-799.

Melixetian, M., A. Ballabeni, L. Masiero, P. Gasparini, R. Zamponi, J. Bartek, J. Lukas and K. Helin (2004a). "Loss of Geminin induces rereplication in the presence of functional p53." J. Cell Biol. **165**(4): 473-482.

Melixetian, M. and K. Helin (2004b). "Geminin: a major DNA replication safeguard in higher eukaryotes." Cell Cycle **3**(8): 1002-1004.

Mendez, J. and B. Stillman (2000). "Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis." Mol Cell Biol **20**(22): 8602-8612.

Mendez, J., X. H. Zou-Yang, S. Y. Kim, M. Hidaka, W. P. Tansey and B. Stillman (2002). "Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication." Mol Cell **9**(3): 481-491.

Meraldi, P. and E. A. Nigg (2001). "Centrosome cohesion is regulated by a balance of kinase and phosphatase activities." J. Cell Sci. **114**(Pt 20): 3749-3757.

Michael, W. M. and J. Newport (1998). "Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1." Science **282**(5395): 1886-1889.

Mimura, S., T. Seki, S. Tanaka and J. F. Diffley (2004). "Phosphorylation-dependent binding of mitotic cyclins to Cdc6 contributes to DNA replication control." Nature **431**(7012): 1118-1123.

Minshull, J., A. Murray, A. Colman and T. Hunt (1991). "*Xenopus* oocyte maturation does not require new cyclin synthesis." J. Cell Biol. **114**(4): 767-772.

Mochida, S., S. Ikeo, J. Gannon and T. Hunt (2009). "Regulated activity of PP2A-B55 delta is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts." EMBO J. **28**(18): 2777-2785.

Mochida, S., S. L. Maslen, M. Skehel and T. Hunt (2010). "Greatwall phosphorylates an inhibitor of protein phosphatase 2A that is essential for mitosis." Science **330**(6011): 1670-1673.

Mochida, S. (2013). "Regulation of alpha-Endosulfine, an inhibitor of protein phosphatase 2A (PP2A), by multisite phosphorylation." FEBS J.

Mori, M., M. Hara, K. Tachibana and T. Kishimoto (2006). "p90Rsk is required for G1 phase arrest in unfertilized starfish eggs." Development.

Mueller, P. R., T. R. Coleman and W. G. Dunphy (1995a). "Cell cycle regulation of a Xenopus wee1-like kinase." Mol. Biol. Cell **6**: 119-134.

Mueller, P. R., T. R. Coleman, A. Kumagai and W. G. Dunphy (1995b). "Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15." Science **270**(5233): 86-90.

Mulner, O., R. Belle and R. Ozon (1983). "cAMP-dependent protein kinase regulates in ovo cAMP level of the *Xenopus* oocyte: evidence for an intracellular feedback mechanism." Mol. Cell. Endocrinol. **31**(1): 151-160.

Mulner, O., D. Huchon, C. Thibier and R. Ozon (1979). "Cyclic AMP synthesis in Xenopus laevis oocytes: inhibition by progesterone." Biochim. Biophys. Acta **582**(1): 179-184.

Murakami, M. S. and G. F. VandeWoude (1998). "Analysis of the early embryonic cell cycles of Xenopus; regulation of cell cycle length by Xe-wee1 and Mos." Development **125**(2): 237-248.

Murakami, M. S., T. D. Copeland and G. F. Vande Woude (1999). "Mos positively regulates Xe-wee1 to lengthen the first mitotic cell cycle of Xenopus." Genes Dev **13**(5): 620-631.

Murray, A. W., M. J. Solomon and M. W. Kirschner (1989). "The role of cyclin synthesis and degradation in the control of maturation promoting factor activity." Nature **339**: 280-286.Murray, A. W. (1992). "Creative blocks: cell-cycle checkponts and feedback controls." Nature **359**: 599-604.

Musacchio, A. and E. D. Salmon (2007). "The spindle-assembly checkpoint in space and time." Nat Rev Mol Cell Biol **8**(5): 379-393.

Nakajima, H., F. Toyoshima-Morimoto, E. Taniguchi and E. Nishida (2003). "Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate." J. Biol. Chem. **278**(28): 25277-25280.

Nakajo, N., T. Oe, K. Uto and N. Sagata (1999). "Involvement of Chk1 Kinase in Prophase I Arrest of Xenopus Oocytes." Dev. Biol. **207**(2): 432-444.

Nakajo, N., S. Yoshitome, J. Iwashita, M. Iida, K. Uto, S. Ueno, K. Okamoto and N. Sagata (2000). "Absence of Wee1 ensures the meiotic cell cycle in Xenopus oocytes." Genes Dev. **14**(3): 328-338.

Narasimhachar, Y. and M. Coue (2009). "Geminin stabilizes CDT1 during meiosis in Xenopus oocytes." J. Biol. Chem.

Narasimhachar, Y., D. R. Webster, D. L. Gard and M. Coue (2012). "Cdc6 is required for meiotic spindle assembly in Xenopus oocytes." Cell Cycle **11**(3): 524-531.

Nebreda, A. R., C. Hill, N. Gomez, P. Cohen and T. Hunt (1993). "The protein kinase mos activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo." FEBS Lett. **333**(1-2): 183-187.

Nebreda, A. R., J. V. Gannon and T. Hunt (1995). "Newly synthesized protein(s) must associate with p34cdc2 to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes." EMBO J. **14**(22): 5597-5607.

Neelsen, K. J., I. M. Zanini, R. Herrador and M. Lopes (2013). "Oncogenes induce genotoxic stress by mitotic processing of unusual replication intermediates." J. Cell Biol. **200**(6): 699-708.

Nguyen, V. Q., C. Co, K. Irie and J. J. Li (2000). "Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7." Curr. Biol. **10**(4): 195-205.

Nguyen, V. Q., C. Co and J. J. Li (2001). "Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms." Nature **411**(6841): 1068-1073.

Niimi, S., S. Arakawa-Takeuchi, B. Uranbileg, J. H. Park, S. Jinno and H. Okayama (2012). "Cdc6 protein obstructs apoptosome assembly and consequent cell death by forming stable complexes with activated Apaf-1 molecules." J. Biol. Chem. **287**(22): 18573-18583.

Nishitani, H. and P. Nurse (1995). "p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast." Cell **83**(3): 397-405.

Nishiyama, A., K. Tachibana, Y. Igarashi, H. Yasuda, N. Tanahashi, K. Tanaka, K. Ohsumi and T. Kishimoto (2000). "A nonproteolytic function of the proteasome is required for the dissociation of Cdc2 and cyclin B at the end of M phase." Genes Dev **14**(18): 2344-2357.

Nishiyama, T., K. Ohsumi and T. Kishimoto (2007). "Phosphorylation of Erp1 by p90rsk is required for cytostatic factor arrest in Xenopus laevis eggs." Nature **446**(7139): 1096-1099.

Norris, R. P., W. J. Ratzan, M. Freudzon, L. M. Mehlmann, J. Krall, M. A. Movsesian, H. Wang, H. Ke, V. O. Nikolaev and L. A. Jaffe (2009). "Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte." Development **136**(11): 1869-1878.

Nurse, P. and Y. Bissett (1981). "Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast." Nature **292**(5823): 558-560.

Oe, T., N. Nakajo, Y. Katsuragi, K. Okazaki and N. Sagata (2001). "Cytoplasmic occurrence of the Chk1/Cdc25 pathway and regulation of Chk1 in Xenopus oocytes." Dev. Biol. **229**(1): 250-261.

Oehlmann, M., A. J. Score and J. J. Blow (2004). "The role of Cdc6 in ensuring complete genome licensing and S phase checkpoint activation." J. Cell Biol. **165**(2): 181-190.

Ohe, M., D. Inoue, Y. Kanemori and N. Sagata (2007). "Erp1/Emi2 is essential for the meiosis I to meiosis II transition in Xenopus oocytes." Dev. Biol. **303**(1): 157-164.

Ohsumi, K., A. Koyanagi, T. M. Yamamoto, T. Gotoh and T. Kishimoto (2004). "Emi1-mediated M-phase arrest in *Xenopus* eggs is distinct from cytostatic factor arrest." Proc. Natl. Acad. Sci. USA.

Okamoto, K., N. Nakajo and N. Sagata (2002). "The existence of two distinct Wee1 isoforms in Xenopus: implications for the developmental regulation of the cell cycle." EMBO J. **21**(10): 2472-2484.

Okazaki, K., K. Hayashida, J. Iwashita, M. Harano, N. Furuno and N. Sagata (1996). "Isolation of a cDNA encoding the X enopus homologue of mammalian Cdc25A that can induce meiotic maturation of oocytes." Gene **178**(1-2): 111-114.

Okumura, E., T. Fukuhara, H. Yoshida, S. Hanada Si, R. Kozutsumi, M. Mori, K. Tachibana and T. Kishimoto (2002). "Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition." Natl. Cell Biol. **4**(2): 111-116.

Okumura, E., A. Morita, M. Wakai, S. Mochida, M. Hara and T. Kishimoto (2014). "Cyclin B-Cdk1 inhibits protein phosphatase PP2A-B55 via a Greatwall kinase-independent mechanism." J. Cell Biol. **204**(6): 881-889.

Ozon, R., R. Belle, D. Huchon and J. Marot (1978). "cAMP-dependent protein kinase and the control of progesterone induced maturation in amphibian oocytes." Ann. Biol. Anim. Biochim. Biophys. **18**: 483-491.

Ozon, R., R. Belle, D. Huchon and O. Mulner (1979a). "Roles of cyclic AMP and calcium in maturation of Xenopus laevis oocytes." Journal of steroid biochemistry **11**(1C): 709-713.

Ozon, R., J. Marot and D. Huchon (1979b). "Progesterone stimulated meiotic maturation in Xenopus laevis: inhibition by methylxanthines " Res. Steroids **8**: 259-263.

Palmer, A., A. C. Gavin and A. R. Nebreda (1998). "A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase myt1." EMBO J. **17**(17): 5037-5047.

Papin, C., C. Rouget, T. Lorca, A. Castro and E. Mandart (2004). "XCdh1 is involved in progesterone-induced oocyte maturation." Dev. Biol. **272**(1): 66-75.

Parker, L. L. and H. Piwnica-Worms (1992). "Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase." Science **257**(5078): 1955-1957.

Pavletich, N. P. (1999). "Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors." J. Mol. Biol. **287**(5): 821-828.

Pedrali-Noy, G., S. Spadari, A. Miller-Faures, A. O. Miller, J. Kruppa and G. Koch (1980). "Synchronization of HeLa cell cultures by inhibition of DNA polymerase alpha with aphidicolin." Nucleic Acids Res. **8**(2): 377-387.

Pelizon, C., M. A. Madine, P. Romanowski and R. A. Laskey (2000). "Unphosphorylatable mutants of Cdc6 disrupt its nuclear export but still support DNA replication once per cell cycle." Genes Dev. **14**(19): 2526-2533.

Peng, A., L. Wang and L. A. Fisher (2011). "Greatwall and Polo-like kinase 1 coordinate to promote checkpoint recovery." J. Biol. Chem. **286**(33): 28996-29004.

Peng, A., T. M. Yamamoto, M. L. Goldberg and J. L. Maller (2010). "A novel role for greatwall kinase in recovery from DNA damage." Cell Cycle **9**(21).

Peng, C. Y., P. R. Graves, R. S. Thoma, Z. Q. Wu, A. S. Shaw and H. PiwnicaWorms (1997). "Mitotic and G(2) checkpoint control: Regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216." Science **277**(5331): 1501-1505.

Peng, C. Y., P. R. Graves, S. Ogg, R. S. Thoma, M. J. Byrnes, 3rd, Z. Wu, M. T. Stephenson and H. Piwnica-Worms (1998). "C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding." Cell Growth Differ. **9**(3): 197-208.

Perkins, G., L. S. Drury and J. F. Diffley (2001). "Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis." EMBO J. **20**(17): 4836-4845.

Peter, M., A. Castro, T. Lorca, C. Le Peuch, L. Magnaghi-Jaulin, M. Doree and J. C. Labbe (2001). "The APC is dispensable for first meiotic anaphase in *Xenopus* oocytes." Natl. Cell Biol. **3**(1): 83-87.

Peter, M., J. C. Labbe, M. Doree and E. Mandart (2002). "A new role for Mos in *Xenopus* oocyte maturation: targeting Myt1 independently of MAPK." Development **129**(9): 2129-2139.

Peters, J. M. (2002). "The anaphase-promoting complex: proteolysis in mitosis and beyond." Mol Cell **9**(5): 931-943.

Petersen, B. O., J. Lukas, C. S. Sorensen, J. Bartek and K. Helin (1999). "Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization." EMBO J. **18**(2): 396-410.

Petersen, B. O., C. Wagener, F. Marinoni, E. R. Kramer, M. Melixetian, E. Lazzerini Denchi, C. Gieffers, C. Matteucci, J. M. Peters and K. Helin (2000). "Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1." Genes Dev **14**(18): 2330-2343.

Piatti, S., C. Lengauer and K. Nasmyth (1995). "Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae." EMBO J. **14**(15): 3788-3799.

Picard, A., G. Peaucellier, F. le Bouffant, C. Le Peuch and M. Doree (1985). "Role of protein synthesis and proteases in production and inactivation of maturation-promoting activity during meiotic maturation of starfish oocytes." Dev. Biol. **109**(2): 311-320.

Polanski, Z., E. Ledan, S. Brunet, S. Louvet, M. H. Verlhac, J. Z. Kubiak and B. Maro (1998). "Cyclin synthesis controls the progression of meiotic maturation in mouse oocytes." Development **125**(24): 4989-4997.

Poon, R. Y., K. Yamashita, J. P. Adamczewski, T. Hunt and J. Shuttleworth (1993). "The cdc2-related protein p40MO15 is the catalytic subunit of a protein kinase that can activate p33cdk2 and p34cdc2." EMBO J. **12**(8): 3123-3132.

Posada, J., N. Yew, N. G. Ahn, G. F. Vande Woude and J. A. Cooper (1993). "Mos stimulates MAP kinase in Xenopus oocytes and activates a MAP kinase kinase in vitro." Mol Cell Biol **13**(4): 2546-2553.

Qian, Y. W., E. Erikson, C. Li and J. L. Maller (1998). "Activated polo-like kinase Plx1 is required at multiple points during mitosis in *Xenopus laevis*." Mol. Cell. Biol. **18**(7): 4262-4271.

Qian, Y. W., E. Erikson, F. E. Taieb and J. L. Maller (2001). "The polo-like kinase plx1 is required for activation of the phosphatase cdc25c and cyclin B-cdc2 in *Xenopus* oocytes." Mol. Biol. Cell **12**(6): 1791-1799.

Rao, P. N. and R. T. Johnson (1970). "Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis." Nature **225**(5228): 159-164.

Rauh, N. R., A. Schmidt, J. Bormann, E. A. Nigg and T. U. Mayer (2005). "Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation." Nature **437**(7061): 1048-1052.

Reimann, J. D. and P. K. Jackson (2002). "Emi1 is required for cytostatic factor arrest in vertebrate eggs." Nature **416**(6883): 850-854.

Reis, A., H. Y. Chang, M. Levasseur and K. T. Jones (2006). "APCcdh1 activity in mouse oocytes prevents entry into the first meiotic division." Natl. Cell Biol. **8**(5): 539-540.

Rempel, R. E., S. B. Sleight and J. L. Maller (1995). "Maternal *Xenopus* Cdk2-cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G1 phase." J. Biol. Chem. **270**(12): 6843-6855.

Remus, D., F. Beuron, G. Tolun, J. D. Griffith, E. P. Morris and J. F. Diffley (2009a). "Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing." Cell **139**(4): 719-730.

Remus, D. and J. F. Diffley (2009b). "Eukaryotic DNA replication control: lock and load, then fire." Curr. Opin. Cell Biol. **21**(6): 771-777.

Reynhout, J. K., C. Taddei, L. D. Smith and M. J. LaMarca (1975). "Response of large oocytes of Xenopus laevis to progesterone in vitro in relation to oocyte size and time after previous HCG-induced ovulation." Dev. Biol. **44**(2): 375-379.

Rhind, N. and D. M. Gilbert (2013). "DNA replication timing." Cold Spring Harb Perspect Biol **5**(8): a010132.

Riedel, C. G., V. L. Katis, Y. Katou, S. Mori, T. Itoh, W. Helmhart, M. Galova, M. Petronczki, J. Gregan, B. Cetin, I. Mudrak, E. Ogris, K. Mechtler, L. Pelletier, F. Buchholz, K. Shirahige and K. Nasmyth (2006). "Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I." Nature **441**(7089): 53-61.

Rime, H., D. Huchon, C. Jessus, J. Goris, W. Merlevede and R. Ozon (1990). "Characterization of MPF activation by okadaic acid in *Xenopus* oocyte." Cell Differ Dev **29**(1): 47-58.

Rime, H., J. Yang, C. Jessus and R. Ozon (1991). "MPF is activated in growing immature Xenopus oocytes in the absence of detectable tyrosine dephosphorylation of P34cdc2." Exp. Cell Res. **196**(2): 241-245.

Rime, H., O. Haccard and R. Ozon (1992). "Activation of p34cdc2 kinase by cyclin is negatively regulated by cyclic amp-dependent protein kinase in Xenopus oocytes." Dev. Biol. **151**(1): 105-110.

Rime, H., D. Huchon, V. Desmedt, C. Thibier, K. Galaktionov, C. Jessus and R. Ozon (1994). "Microinjection of Cdc25 Protein Phosphatase into Xenopus Prophase Oocyte Activates MPF and Arrests Meiosis at Metaphase-I." Biol. Cell **82**(1): 11-22.

Rime, H., C. Jessus and R. Ozon (1995). "Tyrosine phosphorylation of p34cdc2 is regulated by protein phosphatase 2A in growing immature Xenopus oocytes." Exp. Cell Res. **219**(1): 29-38.

Rizzardi, L. F., K. E. Coleman, D. Varma, J. P. Matson, S. Oh and J. G. Cook (2015). "CDK1-dependent inhibition of the E3 ubiquitin ligase CRL4CDT2 ensures robust transition from S Phase to Mitosis." J. Biol. Chem. **290**(1): 556-567.

Rosenblatt, J., Y. Gu and D. O. Morgan (1992). "Human cyclin-dependent kinase-2 is activated during the s-phase and g(2)-phase of the cell cycle and associates with cyclin-a." Proc. Natl. Acad. Sci. USA **89**: 2824-2828.

Rothblum-Oviatt, C. J., C. E. Ryan and H. Piwnica-Worms (2001). "14-3-3 binding regulates catalytic activity of human Wee1 kinase." Cell Growth Differ. **12**(12): 581-589.

Roy, L. M., K. I. Swenson, D. H. Walker, B. G. Gabrielli, R. S. Li, H. Piwnica-Worms and J. L. Maller (1991). "Activation of p34cdc2 kinase by cyclin A." J. Cell Biol. **113**(3): 507-514.

Ruiz, E. J., T. Hunt and A. R. Nebreda (2008). "Meiotic inactivation of Xenopus Myt1 by CDK/XRINGO, but not CDK/cyclin, via site-specific phosphorylation." Mol Cell **32**(2): 210-220.

Ruiz, E. J., M. Vilar and A. R. Nebreda (2010). "A Two-Step Inactivation Mechanism of Myt1 Ensures CDK1/Cyclin B Activation and Meiosis I Entry." Curr. Biol.

Sadler, K. C. and J. V. Ruderman (1998). "Components of the signaling pathway linking the 1-methyladenine receptor to MPF activation and maturation in starfish oocytes." Dev. Biol. **197**(1): 25-38.

Sadler, S. E. and J. L. Maller (1981). "Progesterone inhibits adenylate cyclase in *Xenopus* oocytes. Action on the guanine nucleotide regulatory protein." J. Biol. Chem. **256**(12): 6368-6373.

Sadler, S. E. and J. L. Maller (1983a). "The development of competence for meiotic maturation during oogenesis in Xenopus laevis." Dev. Biol. **98**(1): 165-172.

Sadler, S. E. and J. L. Maller (1983b). "Inhibition of Xenopus oocyte adenylate cyclase by progesterone and 2',5'-dideoxyadenosine is associated with slowing of guanine nucleotide exchange." J. Biol. Chem. **258**(13): 7935-7941.

Sagata, N., M. Oskarsson, T. Copeland, J. Brumbaugh and G. F. Vande Woude (1988). "Function of c-mos proto-oncogene product in meiotic maturation in *Xenopus* oocytes." Nature **335**(6190): 519-525.

Sagata, N., N. Watanabe, G. F. Vande Woude and Y. Ikawa (1989). "The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs." Nature **342**(6249): 512-518.

Sanchez, Y., C. Wong, R. S. Thoma, R. Richman, R. Q. Wu, H. PiwnicaWorms and S. J. Elledge (1997). "Conservation of the Chk1 checkpoint pathway in mammals: Linkage of DNA damage to Cdk regulation through Cdc25." Science **277**(5331): 1497-1501.

Schmidt, A., P. I. Duncan, N. R. Rauh, G. Sauer, A. M. Fry, E. A. Nigg and T. U. Mayer (2005). "Xenopus polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity." Genes Dev **19**(4): 502-513.

Schmitz, M. H., M. Held, V. Janssens, J. R. Hutchins, O. Hudecz, E. Ivanova, J. Goris, L. Trinkle-Mulcahy, A. I. Lamond, I. Poser, A. A. Hyman, K. Mechtler, J. M. Peters and D. W. Gerlich (2010). "Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells." Natl. Cell Biol.

Schultz, R. M. and P. M. Wassarman (1977). "Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse." J. Cell Sci. **24**: 167-194.

Schwab, M., A. S. Lutum and W. Seufert (1997). "Yeast Hct1 is a regulator of Clb2 cyclin proteolysis." Cell **90**(4): 683-693.

Schwab, M. S., B. T. Roberts, S. D. Gross, B. J. Tunquist, F. E. Taieb, A. L. Lewellyn and J. L. Maller (2001). "Bub1 is activated by the protein kinase p90(Rsk) during Xenopus oocyte maturation." Curr. Biol. **11**(3): 141-150.

Senga, T., U. Sivaprasad, W. Zhu, J. H. Park, E. E. Arias, J. C. Walter and A. Dutta (2006). "PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination." J. Biol. Chem. **281**(10): 6246-6252.

Shao, H., R. Li, C. Ma, E. Chen and X. J. Liu (2013). "Xenopus oocyte meiosis lacks spindle assembly checkpoint control." J. Cell Biol.

Sharp-Baker, H. and R. H. Chen (2001). "Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity." J. Cell Biol. **153**(6): 1239-1250.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev **13**(12): 1501-1512.

Shibuya, E. K. and J. V. Ruderman (1993). "Mos induces the in vitro activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells." Mol. Biol. Cell **4**(8): 781-790.

Shirayama, M., W. Zachariae, R. Ciosk and K. Nasmyth (1998). "The polo-like kinase Cdc5p and the WD-repeat protein Cdc2Op/fizzy are regulators and substrates of the anaphase promoting complex in Saccharomyces cerevisiae." EMBO J. **17**(5): 1336-1349.

Shoji, S., N. Yoshida, M. Amanai, M. Ohgishi, T. Fukui, S. Fujimoto, Y. Nakano, E. Kajikawa and A. C. Perry (2006). "Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit via Cdc20." EMBO J. **25**(4): 834-845.

Siddiqui, K., K. F. On and J. F. Diffley (2013). "Regulating DNA replication in eukarya." Cold Spring Harb Perspect Biol **5**(9).

Soewarto, D., H. Schmiady and U. Eichenlaub-Ritter (1995). "Consequences of non-extrusion of the first polar body and control of the sequential segregation of homologues and chromatids in mammalian oocytes." Hum. Reprod. **10**(9): 2350-2360.

Solomon, M. J., J. W. Harper and J. Shuttleworth (1993). "CAK, the p34cdc2 activating kinase, contains a protein identical or closely related to p40MO15." EMBO J. **12**(8): 3133-3142.

Stambrook, P. J. and C. Velez (1976). "Reversible arrest of Chinese hamster V79 cells in G2 by dibutytyl AMP." Exp. Cell Res. **99**(1): 57-62.

Steen, R. L., S. B. Martins, K. Tasken and P. Collas (2000). "Recruitment of protein phosphatase 1 to the nuclear envelope by A-kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly." J. Cell Biol. **150**(6): 1251-1262.

Stemmann, O., H. Zou, S. A. Gerber, S. P. Gygi and M. W. Kirschner (2001). "Dual inhibition of sister chromatid separation at metaphase." Cell **107**(6): 715-726.

Stern, B. and P. Nurse (1996). "A quantitative model for the cdc2 control of S phase and mitosis in fission yeast." Trends Genet. **12**(9): 345-350.

Stinchcomb, D. T., K. Struhl and R. W. Davis (1979). "Isolation and characterisation of a yeast chromosomal replicator." Nature **282**(5734): 39-43.

Stoeber, K., A. D. Mills, Y. Kubota, T. Krude, P. Romanowski, K. Marheineke, R. A. Laskey and G. H. Williams (1998). "Cdc6 protein causes premature entry into S phase in a mammalian cell-free system." EMBO J. **17**(24): 7219-7229.

Strausfeld, U., A. Fernandez, J. P. Capony, F. Girard, N. Lautredou, J. Derancourt, J. C. Labbe and N. J. C. Lamb (1994). "Activation of P34(Cdc2) Protein Kinase by Microinjection of Human Cdc25C into Mammalian Cells - Requirement for Prior Phosphorylation of Cdc25C by P34(Cdc2) at Mitosis on Sites Phosphorylated." J. Biol. Chem. **269**(8): 5989-6000.

Sunkara, P. S., D. A. Wright and P. N. Rao (1979). "Mitotic factors from mammalian cells: a preliminary characterization." Journal of supramolecular structure **11**(2): 189-195.

Swain, J. E., X. Wang, T. L. Saunders, R. Dunn and G. D. Smith (2003). "Specific inhibition of mouse oocyte nuclear protein phosphatase-1 stimulates germinal vesicle breakdown." Mol. Reprod. Dev. **65**(1): 96-103.

Swenson, K. I., K. M. Farrell and J. V. Ruderman (1986). "The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in *Xenopus* oocytes." Cell **47**: 861-870.

Tachibana, K., T. Machida, Y. Nomura and T. Kishimoto (1997). "MAP kinase links the fertilization signal transduction pathway to the G1/S-phase transition in starfish eggs." EMBO J. **16**(14): 4333-4339.

Tachibana, K., M. Mori, T. Matsuhira, T. Karino, T. Inagaki, A. Nagayama, A. Nishiyama, M. Hara and T. Kishimoto (2010). "Initiation of DNA replication after fertilization is regulated by p90Rsk at pre-RC/pre-IC transition in starfish eggs." Proc Natl Acad Sci U S A **107**(11): 5006-5011.

Taieb, F. E., S. D. Gross, A. L. Lewellyn and J. L. Maller (2001). "Activation of the anaphase-promoting complex and degradation of cyclin B is not required for progression from Meiosis I to II in Xenopus oocytes." Curr. Biol. **11**(7): 508-513.

Takeda, N., K. Kyozuka and R. Deguchi (2006). "Increase in intracellular cAMP is a prerequisite signal for initiation of physiological oocyte meiotic maturation in the hydrozoan Cytaeis uchidae." Dev. Biol. **298**(1): 248-258.

Tanaka, T. and M. Yamashita (1995). "Pre-Mpf Is Absent in Immature Oocytes of Fishes and Amphibians except Xenopus." Development Growth & Differentiation **37**(4): 387-393.

Tang, Z. H., T. R. Coleman and W. G. Dunphy (1993). "Two distinct mechanisms for negative regulation of the wee1 protein kinase." EMBO J. **12**: 3427-3436.

Tang, Z., H. Shu, W. Qi, N. A. Mahmood, M. C. Mumby and H. Yu (2006). "PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation." Dev. Cell **10**(5): 575-585.

Tatsumi, Y., S. Ohta, H. Kimura, T. Tsurimoto and C. Obuse (2003). "The ORC1 cycle in human cells: I. cell cycle-regulated oscillation of human ORC1." J. Biol. Chem. **278**(42): 41528-41534.

Terret, M. E., I. Ferby, A. R. Nebreda and M. H. Verlhac (2001). "RINGO efficiently triggers meiosis resumption in mouse oocytes and induces cell cycle arrest in embryos." Biology of the Cell (Paris) **93**(1-2): 89-97.

Thibier, C., V. De Smedt, R. Poulhe, D. Huchon, C. Jessus and R. Ozon (1997). "In vivo regulation of cytostatic activity in *Xenopus* metaphase II- arrested oocytes." Dev. Biol. **185**(1): 55-66.

Thibier, C., O. Mulner and R. Ozon (1982). "In vitro effects of progesterone and estradiol-17 beta on choleragen activated Xenopus oocyte adenylate cyclase." Journal of Steroid Biochemistry **17**(2): 191-196.

Thomas, P., Y. Pang, Y. Zhu, C. Detweiler and K. Doughty (2004). "Multiple rapid progestin actions and progestin membrane receptor subtypes in fish." Steroids **69**(8-9): 567-573.

Thomas, P., Y. Pang, J. Dong, P. Groenen, J. Kelder, J. de Vlieg, Y. Zhu and C. Tubbs (2007). "Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins." Endocrinology **148**(2): 705-718.

Tian, J., H. Gong, G. H. Thomsen and W. J. Lennarz (1997a). "Xenopus laevis sperm-egg adhesion is regulated by modifications in the sperm receptor and the egg vitelline envelope." Dev. Biol. **187**(2): 143-153.

Tian, J., S. Kim, E. Heilig and J. V. Ruderman (2000). "Identification of XPR-1, a progesterone receptor required for *Xenopus* oocyte activation." Proc. Natl. Acad. Sci. USA **97**(26): 14358-14363.

Tian, J., G. H. Thomsen, H. Gong and W. J. Lennarz (1997b). "Xenopus Cdc6 confers sperm binding competence to oocytes without inducing their maturation." Proc Natl Acad Sci U S A **94**(20): 10729-10734.

Tokumoto, M., Y. Nagahama, P. Thomas and T. Tokumoto (2006). "Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation." Gen. Comp. Endocrinol. **145**(1): 101-108.

Toyoshima-Morimoto, F., E. Taniguchi, N. Shinya, A. Iwamatsu and E. Nishida (2001). "Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase." Nature **410**(6825): 215-220.

Tso, J., C. Thibier, O. Mulner and R. Ozon (1982). "Microinjected progesterone reinitiates meiotic maturation of Xenopus laevis oocytes." Proc. Natl. Acad. Sci. USA **79**(18): 5552-5556.

Tung, J. J., D. V. Hansen, K. H. Ban, A. V. Loktev, M. K. Summers, J. R. Adler, 3rd and P. K. Jackson (2005). "A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of *Xenopus* eggs." Proc. Natl. Acad. Sci. USA.

Tung, J. J., K. Padmanabhan, D. V. Hansen, J. D. Richter and P. K. Jackson (2007). "Translational unmasking of Emi2 directs cytostatic factor arrest in meiosis II." Cell Cycle **6**(6): 725-731.

Tunquist, B. J., P. A. Eyers, L. G. Chen, A. L. Lewellyn and J. L. Maller (2003). "Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor-mediated metaphase arrest." J. Cell Biol. **163**(6): 1231-1242.

Uhlmann, F. and K. Nasmyth (1998). "Cohesion between sister chromatids must be established during DNA replication." Curr. Biol. **8**(20): 1095-1101.

Vagnarelli, P., S. Ribeiro, L. Sennels, L. Sanchez-Pulido, F. de Lima Alves, T. Verheyen, D. A. Kelly, C. P. Ponting, J. Rappsilber and W. C. Earnshaw (2011). "Repo-Man coordinates chromosomal reorganization with nuclear envelope reassembly during mitotic exit." Dev. Cell **21**(2): 328-342.

Verlhac, M., J. Kubiak, M. Weber, G. Geraud, W. Colledge, M. Evans and B. Maro (1996a). "Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse." Development **122**(3): 815-822.

Verlhac, M. H., J. Z. Kubiak, M. Weber, G. Geraud, W. H. Colledge, M. J. Evans and B. Maro (1996b). "Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse." Development **122**(3): 815-822.

Verlhac, M. H., C. Lefebvre, J. Z. Kubiak, M. Umbhauer, P. Rassinier, W. Colledge and B. Maro (2000). "Mos activates MAP kinase in mouse oocytes through two opposite pathways." EMBO J. **19**(22): 6065-6074.

Vigneron, S., E. Brioudes, A. Burgess, J. C. Labbe, T. Lorca and A. Castro (2009). "Greatwall maintains mitosis through regulation of PP2A." EMBO J. **28**(18): 2786-2793.

Virsolvy-Vergine, A., H. Leray, S. Kuroki, B. Lupo, M. Dufour and D. Bataille (1992). "Endosulfine, an endogenous peptidic ligand for the sulfonylurea receptor: purification and partial characterization from ovine brain." Proc. Natl. Acad. Sci. USA **89**(14): 6629-6633.

Visintin, R., S. Prinz and A. Amon (1997). "CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis." Science **278**(5337): 460-463.

Von Stetina, J. R., S. Tranguch, S. K. Dey, L. A. Lee, B. Cha and D. Drummond-Barbosa (2008). "alpha-Endosulfine is a conserved protein required for oocyte meiotic maturation in Drosophila." Development **135**(22): 3697-3706.

Walaas, S. I., D. W. Aswad and P. Greengard (1983). "A dopamine- and cyclic AMP-regulated phosphoprotein enriched in dopamine-innervated brain regions." Nature **301**(5895): 69-71.

Wang, J. and X. J. Liu (2004). "Progesterone inhibits protein kinase A (PKA) in *Xenopus* oocytes: demonstration of endogenous PKA activities using an expressed substrate." J. Cell Sci. **117**(Pt 21): 5107-5116.

Wang, L., Q. Guo, L. A. Fisher, D. Liu and A. Peng (2015). "Regulation of polo-like kinase 1 by DNA damage and PP2A/B55alpha." Cell Cycle **14**(1): 157-166.

Wang, R., G. He, M. Nelman-Gonzalez, C. L. Ashorn, G. E. Gallick, P. T. Stukenberg, M. W. Kirschner and J. Kuang (2007). "Regulation of Cdc25C by ERK-MAP Kinases during the G(2)/M Transition." Cell **128**(6): 1119-1132.

Wang, R., S. Y. Jung, C. F. Wu, J. Qin, R. Kobayashi, G. E. Gallick and J. Kuang (2010). "Direct roles of the signaling kinase RSK2 in Cdc25C activation during Xenopus oocyte maturation." Proc Natl Acad Sci USA **107**(46): 19885-19890.

Wasch, R. and F. R. Cross (2002). "APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit." Nature **418**(6897): 556-562.

Wassarman, P. M., D. F. Albertini, W. J. Josefowicz and G. E. Letourneau (1976a). "Cytochalasin B-induced pseudo-cleavage of mouse oocytes in vitro: asymmetric localization of mitochondria and microvilli associated with a stage-specific response." J. Cell Sci. **21**(3): 523-535.

Wassarman, P. M., W. J. Josefowicz and G. E. Letourneau (1976b). "Meiotic maturation of mouse oocytes in vitro: inhibition of maturation at specific stages of nuclear progression." J. Cell Sci. **22**(3): 531-545.

Wasserman, W. J. and Y. Masui (1974). "A study on gonadotropin action in the induction of oocyte maturation in Xenopus laevis." Biol. Reprod. **11**(2): 133-144.

Wasserman, W. J. and Y. Masui (1975). "Effects of cyclohexamide on a cytoplasmic factor initiating meiotic naturation in Xenopus oocytes." Exp. Cell Res. **91**(2): 381-388.

Watanabe, N., H. Arai, Y. Nishihara, M. Taniguchi, N. Watanabe, T. Hunter and H. Osada (2004). "M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP." Proc Natl Acad Sci U S A **101**(13): 4419-4424.

Watanabe, N., M. Broome and T. Hunter (1995). "Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle." EMBO J. **14**(9): 1878-1891.

Watanabe, N., G. F. Vande Woude, Y. Ikawa and N. Sagata (1989). "Specific proteolysis of the c-mos proto-oncogene product by calpain on fertilization of *Xenopus* eggs." Nature **342**(6249): 505-511.

Weinert, T. A. and L. H. Hartwell (1988). "The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*." Science **241**: 317-322.

Weinreich, M., C. Liang, H. H. Chen and B. Stillman (2001). "Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle." Proc Natl Acad Sci U S A **98**(20): 11211-11217.

Weinreich, M., C. Liang and B. Stillman (1999). "The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin." Proc Natl Acad Sci U S A **96**(2): 441-446.

Weintraub, H., M. Buscaglia, M. Ferrez, S. Weiller, A. Boulet, F. Fabre and E. E. Baulieu (1982). "[Demonstration of maturation promoting factor activity in Saccharomyces cerevisiae]." Comptes rendus des seances de l'Academie des sciences. Serie III, Sciences de la vie **295**(13): 787-790.

Wells, N. J., N. Watanabe, T. Tokusumi, W. Jiang, M. A. Verdecia and T. Hunter (1999). "The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression." J. Cell Sci. **112 (Pt 19)**: 3361-3371.

Whitmire, E., B. Khan and M. Coue (2002). "Cdc6 synthesis regulates replication competence in Xenopus oocytes." Nature **419**(6908): 722-725.

Williams, B. C., J. J. Filter, K. A. Blake-Hodek, B. E. Wadzinski, N. J. Fuda, D. Shalloway and M. L. Goldberg (2014). "Greatwall-phosphorylated Endosulfine is both an inhibitor and a substrate of PP2A-B55 heterotrimers." eLife **3**: e01695.

Williams, R. S., R. V. Shohet and B. Stillman (1997). "A human protein related to yeast Cdc6p." Proc Natl Acad Sci U S A **94**(1): 142-147.

Winston, N. J. (1997). "Stability of cyclin B protein during meiotic maturation and the first mitotic cell division in mouse oocytes." Biol. Cell **89**(3): 211-219.

Wolthuis, R., L. Clay-Farrace, W. van Zon, M. Yekezare, L. Koop, J. Ogink, R. Medema and J. Pines (2008). "Cdc20 and Cks direct the spindle checkpoint-independent destruction of cyclin A." Mol Cell **30**(3): 290-302.

Wu, J. Q., J. Y. Guo, W. Tang, C. S. Yang, C. D. Freel, C. Chen, A. C. Nairn and S. Kornbluth (2009). "PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation." Natl. Cell Biol. **11**(5): 644-651.

Wu, J. Q., D. V. Hansen, Y. Guo, M. Z. Wang, W. Tang, C. D. Freel, J. J. Tung, P. K. Jackson and S. Kornbluth (2007a). "Control of Emi2 activity and stability through Mos-mediated recruitment of PP2A." Proc. Natl. Acad. Sci. USA.

Wu, Q., Y. Guo, A. Yamada, J. A. Perry, M. Z. Wang, M. Araki, C. D. Freel, J. J. Tung, W. Tang, S. S. Margolis, P. K. Jackson, H. Yamano, M. Asano and S. Kornbluth (2007b). "A Role for Cdc2- and PP2A-Mediated Regulation of Emi2 in the Maintenance of CSF Arrest." Curr. Biol. **17**(3): 213-224.

Wurzenberger, C., M. Held, M. A. Lampson, I. Poser, A. A. Hyman and D. W. Gerlich (2012). "Sds22 and Repo-Man stabilize chromosome segregation by counteracting Aurora B on anaphase kinetochores." J. Cell Biol. **198**(2): 173-183.

Xouri, G., A. Squire, M. Dimaki, B. Geverts, P. J. Verveer, S. Taraviras, H. Nishitani, A. B. Houtsmuller, P. I. Bastiaens and Z. Lygerou (2007). "Cdt1 associates dynamically with chromatin throughout G1 and recruits Geminin onto chromatin." EMBO J. **26**(5): 1303-1314.

Yamamoto, T. M., K. Blake-Hodek, B. C. Williams, A. L. Lewellyn, M. L. Goldberg and J. L. Maller (2011). "Regulation of Greatwall kinase during *Xenopus* oocyte maturation." Mol. Biol. Cell **22**(13): 2157-2164.

Yamashita, M., H. Kajiura, T. Tanaka, S. Onoe and Y. Nagahama (1995). "Molecular mechanisms of the activation of maturation-promoting factor during goldfish oocyte maturation." Dev. Biol. **168**: 62-75.

Yan, H., S. Gibson and B. K. Tye (1991). "Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function." Genes Dev **5**(6): 944-957.

Yang, J., K. Winkler, M. Yoshida and S. Kornbluth (1999). "Maintenance of G2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import." EMBO J. **18**(8): 2174-2183.

Yew, N., M. L. Mellini and G. F. Vande Woude (1992). "Meiotic initiation by the mos protein in *Xenopus*." Nature **355**(6361): 649-652.

Yim, H. and R. L. Erikson (2010). "Cell division cycle 6, a mitotic substrate of polo-like kinase 1, regulates chromosomal segregation mediated by cyclin-dependent kinase 1 and separase." Proc Natl Acad Sci U S A **107**(46): 19742-19747.

Yim, H., I. S. Hwang, J. S. Choi, K. H. Chun, Y. H. Jin, Y. M. Ham, K. Y. Lee and S. K. Lee (2006). "Cleavage of Cdc6 by caspase-3 promotes ATM/ATR kinase-mediated apoptosis of HeLa cells." J. Cell Biol. **174**(1): 77-88.

Yim, H., Y. H. Jin, B. D. Park, H. J. Choi and S. K. Lee (2003). "Caspase-3-mediated cleavage of Cdc6 induces nuclear localization of p49-truncated Cdc6 and apoptosis." Mol. Biol. Cell **14**(10): 4250-4259.

Yoshida, K., N. Sugimoto, S. Iwahori, T. Yugawa, M. Narisawa-Saito, T. Kiyono and M. Fujita (2010). "CDC6 interaction with ATR regulates activation of a replication checkpoint in higher eukaryotic cells." J. Cell Sci. **123**(Pt 2): 225-235.

Yu, J., Y. Zhao, Z. Li, S. Galas and M. L. Goldberg (2006). "Greatwall kinase participates in the Cdc2 autoregulatory loop in Xenopus egg extracts." Mol. Cell **22**(1): 83-91.

Zeng, Y., K. C. Forbes, Z. Wu, S. Moreno, H. Piwnica-Worms and T. Enoch (1998). "Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1." Nature **395**(6701): 507-510.

Zheng, X. F. and J. V. Ruderman (1993). "Functional Analysis of the P-Box, a Domain in Cyclin-B Required for the Activation of Cdc25." Cell **75**(1): 155-164.

Zhou, Y., Y. P. Ching, R. W. Ng and D. Y. Jin (2002). "The APC regulator CDH1 is essential for the progression of embryonic cell cycles in Xenopus." Biochem. Biophys. Res. Commun. **294**(1): 120-126.

Zhu, L., N. Ohan, Y. Agazie, C. Cummings, S. Farah and X. J. Liu (1998). "Molecular cloning and characterization of *Xenopus* insulin-like growth factor-1 receptor: Its role in mediating insulin-induced *Xenopus* oocyte maturation and expression during embryogenesis." Endocrinology **139**(3): 949-954.

Zhu, Y., J. Bond and P. Thomas (2003). "Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor." Proc. Natl. Acad. Sci. USA **100**(5): 2237-2242.

Zhu, Y., D. Liu, Z. C. Shaner, S. Chen, W. Hong and E. J. Stellwag (2015). "Nuclear progestin receptor (pgr) knockouts in zebrafish demonstrate role for pgr in ovulation but not in rapid non-genomic steroid mediated meiosis resumption." Frontiers in endocrinology **6**: 37.

RESUME

L'objectif de cette thèse a été de comprendre deux caractéristiques majeures des divisions méiotiques chez la femelle: le blocage en prophase de 1ère division méiotique qui permet à l'ovocyte d'accumuler des réserves énergétiques et des déterminants nécessaires au développement embryonnaire ; et l'absence de phase-S entre les deux divisions méiotiques ce qui permet de former des cellules haploïdes aptes à la fécondation. Pour cela, j'ai choisi comme modèle d'étude l'ovocyte de Xénope qui permet de suivre ces processus *in vitro* en réponse à la progestérone. L'ovocyte subit les deux divisions méiotiques grâce à l'activation du facteur universel de la division cellulaire, le MPF, et se bloque en métaphase de 2ème division méiotique dans l'attente d'être fécondé. Chez tous les vertébrés, le 1er arrêt en prophase dépend de l'activité de la protéine kinase dépendante de l'AMPc, PKA, dont l'inactivation est nécessaire pour la reprise de la méiose. Le substrat de PKA dans l'ovocyte était resté inconnu. Nous avons découvert que la protéine Arpp19, jusqu'alors connue pour son rôle positif dans l'activation du MPF, est phosphorylée par PKA de cette phosphorylation bloque l'activation du MPF nécessaire pour la levée du blocage en prophase. ARPP19 possède donc un double rôle, le 1er exercé comme substrat de PKA et responsable de l'arrêt en prophase, le second dans l'activation du MPF suite à un changement dans sa phosphorylation. Dans un second temps, nous avons étudié la protéine Cdc6, un acteur majeur de la réplication de l'ADN. Absente en prophase, Cdc6 s'accumule entre les deux divisions méiotiques ce qui permet à l'ovocyte d'acquérir la compétence à répliquer l'ADN. Cette compétence ne s'exprime pas ce qui permet de réduire de moitié la ploïdie. Nous avons montré que Cdc6 est un inhibiteur puissant du MPF capable de bloquer les divisions méiotiques et d'induire la réplication de l'ADN. Pour éviter ces effets délétères l'accumulation de Cdc6 est strictement régulée lors des deux divisions méiotiques, ce qui est absolument requis pour assurer l'enchainement des deux divisions cellulaires sans phase-S intercalaire.

ABSTRACT

The goal of my PhD project was to understand two main features of the female meiotic division: the arrest in prophase of the 1st meiotic division that allows the accumulation of nutrients and determinants necessary for the embryonic cell cycles; and the absence of S-phase between the two meiotic divisions in order to produce haploid gametes. For this purpose, I studied *Xenopus* oocytes, a powerful model system that allows the biochemical analysis of these two processes *in vitro*. In ovary, oocytes are arrested in prophase I and resume meiosis in response to progesterone. The oocytes then proceed through the 1st and the 2nd meiotic divisions and halt at metaphase II, awaiting for fertilization. These two consecutive divisions are controlled by two waves of Cdk1 activation, the universal factor responsible for the entry into mitosis. I analysed the mechanisms responsible for arresting the oocyte in prophase I. In all vertebrates, this arrest depends on a high activity of the cAMP-dependent protein kinase, PKA, whose downregulation is required for the release of the prophase block. The substrate of PKA had never been identified up to date. I discovered that the small protein Arpp19, already known for positively regulating entry into M-phase, is phosphorylated by PKA in prophase I and is dephosphorylated upon progesterone addition, an event required for Cdk1 activation. Hence, Arpp19 has a dual function, responsible of the prophase arrest as a PKA substrate, and then converted into an activator of Cdk1 by changes of its phosphorylation pattern. The second part of my thesis has been dedicated to understanding the role and the regulation of the Cdc6 protein during meiotic divisions. This protein is essential for DNA replication in somatic cells. It is accumulated between the two oocyte meiotic divisions and restores the competence to replicate DNA in oocyte. However, this competence is repressed before fertilization, allowing formation of haploid cells. I found that the accumulation of Cdc6 is tightly controlled during meiotic maturation by the Cyclin B accumulation and the Mos/MAPK pathway. I further demonstrated that Cdc6 is a strong

inhibitor of Cdk1 in *Xenopus* oocytes and that the timely accumulation of Cdc6 is required to coordinate the two meiotic divisions with no intercaling S-phase.